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Effect of Female Sex Hormones, Thyroxine and Vitamin B_{12} on Embryonic Mortality in Rats

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ABSTRACT

This investigation explored the possibility of reducing prenatal death losses by treating animals with exogenous progesterone and estrogen, thyroxine, vitamin B_{12}, and various combinations of these agents to produce a better intra-uterine environment. Hundred-day-old CNF virgin female rats were used in two separate studies. Prenatal death losses were determined by subtracting the number of living fetuses in the uterus from the number of corpora lutea in the ovary when the females were sacrificed on the 15th day of pregnancy. Uterine growth was measured by the amount of DNA in the uterine tissue.

In the first experiment, 45 female rats treated with estradiol and progesterone at the ratio of 1:1000 or 1:2000 had significantly larger litters ($P<.025$) and fewer missing embryos than females in the control group ($P<.005$). The differences in DNA concentration of the uteri among the three groups were not significant ($P<.10$).

In the second experiment 54 female rats were used and estrogen and progesterone (1:2000) were administered with and without L-thyroxine and vitamin B_{12}. Again it was noted that the estradiol and progesterone treatment significantly increased litter size at the 15th day of pregnancy over the control females and significantly reduced the prenatal death losses. In this experiment the DNA content of the uterine tissue was significantly higher in females treated with these two hormones than in non-treated females, indicating true growth of the uterine tissue. Exogenous L-thyroxine and vitamin B_{12} failed to affect uterine growth or prenatal losses.

The thyroid secretion rate (TSR) was determined in 54 female rats at a weight of approximately 180 grams by the I^{131} technique. Little correlation was noted between the TSR and reproductive performance.

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INTRODUCTION

Fertility involves all physiological, genetic, and environmental factors, in both male and female, which enhance or inhibit the production of the maximum number of viable offspring resulting from a single mating.

In litter-bearing animals, such as swine, we are confronted with a problem peculiar to these animals. To assure the uterus will be utilized at its maximum, the number of ova produced during a single ovulation period exceeds the number of embryos which the uterus is capable of maintaining throughout gestation. In most litter-bearing animals, including swine, only two-thirds of the ova shed during an ovulation period produce viable young at parturition. Hence, approximately one-third of the potential pig crop is lost. Litter size in swine or other litter-bearing animals, then, is dependent upon three physiological reproductive processes: ovulation rate, the number of ova shed which are fertilized, and the number of fertilized ova maintained throughout gestation by the uterus. Previous work in this laboratory has shown that at least 95 percent of ova shed are fertilized; therefore, in most cases failures of fertilization are of minor importance. Since an excess of ova are produced by each sow, efforts to increase ovulation rate must await the solution of our chief problem, the uterus as an environment for the developing embryos.

Our primary problem, then, concerns the uterus. Essentially, we are confronted with the problem of improving upon nature by enhancing the inherent potentialities of the uterus to maintain viable embryos during gestation. Uterine size or the amount of intra-uterine space per developing embryo is important in providing an optimum intra-uterine environment as shown in previous investigations in this laboratory. In addition, and probably of more importance, are the physiological, anatomical and biochemical changes induced in uterine tissues by hormones, vitamins, and other physiological agents. Estrogen and progesterone administered in the proper ratio to sows or gilts during a ten-day period in gestation favorably influence both uterine size and the intra-uterine environment. However, more information is needed regarding these hormones and their influence upon uterine physiology. Little information is available on the role of the thyroid hormone upon the uterus. Vitamins and other physiological agents may also play an important role in uterine physiology.

The present investigation is a series of pilot experiments utilizing rats to provide information and develop methods for future research with swine. The

purposes are: (1) to study the fundamental anatomy and physiology of the reproductive organs during the puberal age and gestational period and (2) to explore the possibility of insuring a better intra-uterine environment, and thus prevent prenatal loss, by means of exogenous progesterone and estrogen, thyroxine, vitamin B_{12} and various combinations of these exogenous agents. An endeavor was made to combine as nearly as possible all factors involved in creating an optimal intra-uterine environment for the embryos during pregnancy.

It was hoped that results of this fundamental research would also yield information leading to practical applications in swine production and provide breeders with knowledge leading to the development of indices for the selection of females capable of producing litters of maximum size.

REVIEW OF LITERATURE

Embryonic Mortality

Recognition of the importance of prenatal death in livestock production was given special emphasis by Hammond (1914). He examined seven sows in various stages of pregnancy and found that the number of normal fetuses was 73 percent of the number of corpora lutea on both ovaries. Additional data presented by Hammond (1921) placed the number of normal fetuses as 67 percent of the number of existing corpora lutea. In observations on 80 pregnant ewes, 13 percent of the ova were not represented by viable embryos. The numbers of atrophic fetuses and missing eggs were approximately equal. Hammond cited four major factors contributing to the embryonic mortality:

1. Limited capacity of the uterus.
2. Nutritional deficiency.
3. Bacterial infection.
4. Innate lack of viability in the fetus.

Henning (1939) estimated the incidence of fetal mortality in sheep and found that 16 percent of the corpora lutea were not accounted for by live fetuses. Henning was the first investigator to cite that an increase in the number of ova shed resulted in an increased embryonic mortality.

That the probability of embryonic death may differ from litter to litter was reported by Brambell (1948) on the basis of data obtained with wild rabbits.

A survey of the literature suggests that an estimate of embryonic death requires an embryo census at two successive stages of gestation and perhaps on two separate but entirely comparable groups of animals. The two stages of gestation are immediately after fertilization and again at term. The first gives an estimate of the ovulation rate and the fertilization rate and the second gives an estimate of the mortality rate of fertilized ova and developing embryos. However, on the basis of investigations in this laboratory on swine, Squiers *et al.* (1952) and Lerner *et al.* (1958), two stages of embryonic mortality were disclosed: one occurring at a critical stage prior to the twenty-fifth day and a second occurring between this early period and parturition. The major portion of the deaths occur during the early stage.

Reevaluation for swine of the proportion of corpora lutea accounted for by young born at term was made by Casida (1951) and found to be 56 percent. This evaluation was based on the comparison of similar groups of females; one group was slaughtered and examined shortly after fertilization and the other group was allowed to farrow. This figure compares favorably with that of Squiers *et al.* (1952) who estimated in a similar manner that 54 percent of the ova ovulated were represented by living pigs at parturition.

Abnormalities of fertilized bovine ova were reported by Winters *et al.* (1942) and they cited the possible role of these abnormalities in lowered fertility in cattle. Embryonic death was demonstrated as an important cause of lowered fertility in cattle by Laing (1949). His data on 11 maiden heifers inseminated with the semen of a single bull of high fertility showed a fertilization rate of 100 percent.

A later attempt was made to determine the fertilization rate in normal first-service heifers inseminated artificially and to relate this to the non-return rate in the field in order to estimate embryonic death. The results reported by Kidder *et al.* (1952) indicate a fertilization rate of approximately 86 percent and an embryonic death rate of about 30 percent. Cows bred to the bulls of low fertility had a higher embryonic death rate than those bred to bulls which were more fertile.

Estimates of embryonic death rate made for the rabbit, both wild (Brambell, 1948) and domestic (Casida, 1951), appear to be in line with those for swine, 35 to 40 percent.

An extensive literature exists on the influence of nutrition upon embryonic survival. Since it is not pertinent to this investigation it will not be reviewed.

Inbreeding is another factor with an effect upon embryonic death rate. Squiers *et al.* (1952) reported on the effects of inbreeding upon embryonic death in swine. They estimated that, for each 10 percent of inbreeding of the parent lines, the advantage for line-cross sows amounted to 0.33 more pigs at the twenty-fifth day of gestation although both inbred and line-cross animals were carrying outbred pigs. They also stressed the point that embryonic death was of more importance in controlling litter size in swine than ovulation rate. The standard partial regression of litter size on ovulation rate was 0.79 whereas on mortality rate it was 0.94.

Analyses of the potential causes of embryonic death have been made by a number of investigators. Hammond (1914) tended to eliminate disease as a factor because dead and live embryos existed side by side in the same uterus and bacteria usually were not found in the uteri. Hammond was also of the opinion that general nutrition of the mother was not a major factor. Hammond concluded that some factor inherent in the ovum seemed to be the most likely cause. Since the embryonic death rate increased with an increase in the ovulation rate, he suspected that ovarian nutrition was a limiting factor.

In more recent investigations the search has continued for potential causes of embryonic death or for associated phenomena which may have the potential

to cause or influence embryonic death or which may lead us to the causative factors. In an endeavor to discover associated phenomena related to embryonic mortality, Asdell *et al.* (1942) and Bentley *et al.* (1951) studied the blood chemistry of repeat breeding cows but found little that was unusual. Another associated phenomenon studied was the defense mechanism of the uterus against invading pathological organisms. The defense mechanism of the uterus of the repeat breeding cow seems to differ from that of a first service cow. This phenomenon was studied by Block *et al.* (1953) who reported that the uterine defense mechanism against infection is about as effective in the ovariectomized animal as in the estrogen-treated animal. Progesterone, on the other hand, appears to block the natural defense mechanism in the tissues.

As a result of a series of investigations at the Missouri station over a period of 15 years with rats and swine, it can be concluded that the ovarian or so-called pregnancy hormones, progesterone and estrogen, are the major factors influencing the embryonic death rate and thus determining litter size. Further, as the following brief review of this work will demonstrate, these two hormones produce their influence upon embryonic deaths (or conversely upon embryonic survival) indirectly through their effect upon uterine growth and uterine physiological activities, thus producing a favorable or unfavorable intra-uterine environment for the embryos.

The initial and extensive investigation by Squiers *et al.* (1952) with sows and gilts resulted in the following conclusive findings:

1. Fertilization is not a major causative factor in embryonic mortality.
2. Gross anatomical abnormalities of the genital organs of the sow are of but minor importance since their occurrence is rather rare.
3. The embryonic mortality rate increases with ovulation rate. In gilts with a lower ovulation rate than sows, the embryonic mortality rate is lower but on a percentage basis (ova shed vs. number of dead embryos) the embryonic mortality is similar.
4. The major portion of the embryonic mortality occurs prior to the 25th day of the gestation period and it is this embryo loss which determines litter size.

In a subsequent investigation, Lerner *et al.* (1958), an endeavor was made to determine the exact time of embryonic mortality during the early stages of gestation. However, before the 17th day of gestation it was difficult to determine the exact status of the embryos. The results of this investigation showed that of a total embryonic mortality of 33.63 percent at the 25th day, 25.1 percent had occurred prior to the 17th day.

That uterine capacity or size of the uterus has a marked influence upon embryonic mortality was shown by Rathnasabapathy *et al.* (1956). It was found that 350 to 450 mm. of uterine space per embryo is the optimum space for the fullest growth of embryos examined at the 55th day of gestation in Landrace x Poland cross bred gilts. The comparative study of the size of the reproductive organs at different stages in the life of the gilts indicates that an increase of 292

percent in the length of the uterus had taken place from 169 days of age (200 pounds body weight) to the middle of pregnancy or at about 257 days of age. This optimum space will then be represented by 120 to 154 mm. of uterine length per ovum at 169 days of age. Hence, hormones or other factors which control uterine growth, indirectly influence the rate of embryonic mortality.

Embryonic mortality is essentially a phenomenon of early pregnancy as attested by the results of the research at the Missouri Agricultural Experiment Station cited previously. Hence, the first three or four weeks in the sow is a critical period during which some factor of import to the uterine tissues may be involved.

Results of experiments with rats by Bredeck and Mayer (1955) suggested that the reproductive hormones, especially progesterone, play an important role in the regulation of the activity rate of uterine phosphatases and possibly other uterine enzymes. An investigation was also made of the qualitative and quantitative aspects of the metabolites of progesterone and progesterone-like steroids in the urine of sows combined with a study of the relationship of these metabolites to embryonic mortality during early pregnancy. Mayer *et al.* (1961) reported the results of these investigations in which sows employed by Squiers *et al.* (1952) were utilized as experimental animals. Both conjugated and non-conjugated urinary metabolites were determined by a method developed in our laboratory. A close relationship was found between the excretion rate of the conjugated and non-conjugated metabolites. Data were obtained suggesting that the precursor of a conjugated non-ketonic steroid in the urine was produced by the corpora lutea in early pregnancy and that the placenta and/or other tissues produced this precursor after the 24th day of gestation. On the basis of these results it appears that during a critical stage in early pregnancy in the sow, when the embryonic mortality rate is the highest, a precursor of the conjugated pregnanediol metabolite ceases to be produced by one tissue and its production is taken over by another tissue. The urinary analyses show that at this period the excretion rate of this metabolite is at the lowest level of the gestation period.

The results of a study by Gawienowski and Mayer (1957), in which they determined the progesterone content of ovarian tissues and adrenal glands, indicate that progesterone is the precursor of the important metabolite under discussion. The concentration of the urinary metabolites is significantly correlated with the number of embryos implanted and with the percentage of the ova shed which were implanted. These significant correlations point to the significant role of progesterone or other progestins in embryonic mortality during early prognancy.

In a series of pilot experiments with rats at the Missouri Agricultural Experiment Station (unpublished data), in which the females were ovariectomized four days after mating, results were obtained which showed that a mixture of progesterone and estrogen was more efficient in the maintenance of pregnancy than either hormone alone. Further, it was shown that the best results were obtained when the hormones were in the mixture in the proper ratio of 1 part of

estrogen to 2000 parts of progesterone. Dosage levels for pregnancy maintenance in ovariectomized rats were also established.

With the results obtained in the investigations described above as a guide, Reddy *et al.* (1958) made an extensive investigation with gilts and sows as the experimental animals. After many trials they found that a progesterone-estrogen therapy in the form of daily injections of 25 mg. of progesterone plus 12.5 ug. of estrone (ratio of 2000:1) per gilt for ten consecutive days beginning the 14th day of gestation would provide a more favorable uterine environment and thus limit the embryonic mortality to 13.51 percent. The treated animals had larger uteri, the embryos were more uniformly spaced and were more equal in size than those of the control animals.

There are numerous reports in the literature on the therapeutic use of progesterone and estrogen or progesterone alone in pregnancy maintenance in a variety of experimental animals. Burdick *et al.* (1941) worked with mice and observed that 1 mgm. of progesterone administered daily beginning on the day of mating prevented implantation. Similar treatment beginning the second day after mating did not inhibit implantation and the embryos continued to grow during the period of treatment. Death of the embryos occurred upon cessation of the treatment. Lyons (1943) successfully maintained pregnancy in hypophysectomized-oophoretomized rats by daily injections of 1 ug. of estrone plus 3-4 mgm. of progesterone.

Early mortality was decreased by treatment of female rabbits with progesterone and estrogen in combination. The investigators, Warwick *et al.* (1943), stated, "The most logical assumption is that they (estrogen and progesterone) improved the uterine environment and thereby prolonged the life of the embryos.

It must be emphasized that both the quantity of hormones and the ratio of the two hormones (estrogen and progesterone) administered are critical factors in determining the success of this type of hormone therapy in the pregnant female. The detrimental effects of unphysiological doses of progesterone are evident in the results of work reported by Sammelwitz *et al.* (1956) in which progesterone was injected into gilts at levels of 50, 100, 200 and 400 mgm. daily for 26 days beginning the day after mating. In some gilts of the 200-mgm.-treated group and in all of the gilts of the 400-mgm.-treated group the corpora lutea were completely degenerated by the time of autopsy. In addition, the embryonic survival in the 60 and 100-mgm. groups was lower than that in the controls.

Herrick (1953) reported that 35 percent of a group of repeat breeder cows settled on the first service following progesterone therapy in contrast to the 5 percent settling rate in non-treated controls. In similar work by Dawson (1954) 47 percent of the control cows settled.

That the effectiveness of combined estrogen-progesterone therapy in ovariectomized gilts varied considerably with the ratio of the two hormones and with the level administered at ratios of 1:100, 1:1000 and 1:2000 was shown by Day (1959) at the Missouri Agricultural Experiment Station. Results obtained with

different dosage levels and ratios in the initial phase of his investigation indicated that the daily injection of 50 ug. of estradiol benzoate and 100 mg. of progesterone, a ratio of 1:2000, was highly effective in inducing optimum uterine conditions for embryonic survival.

Role of the Thyroid Hormone in Reproductive Physiology of the Female

Although numerous investigations conducted over a period of more than half a century, indicate that the thyroid hormone is implicated in some manner in the reproductive physiology of the female, the precise role that it plays has not been clarified. An analysis of the information available indicates the presence of nicely balanced interaction's between the hormones of the thyroid, pituitary, and the ovaries. An excess or deficiency of one or more hormones of these glands will result, in most cases, in impaired reproductive function.

Gudernatsch (1915) published the earliest report on the effect of the thyroid gland upon reproductive processes in the female. He fed female rats with fresh beef thyroid gland and reported that the rats refused to breed under these conditions. Pregnancies occurred only after thyroid gland feeding was discontinued and then they were accompanied by abortion, death of offspring soon after birth, or by retarded growth in the surviving young. The results of Evans and Long (1921) appeared to contradict those of Gudernatsch. They observed that feeding of thyroid tissue in doses of ¼ to 1½ gm. daily caused little disturbance of estrous cycles.

A severe hyperthyroidism was induced in female rabbits by Kunde *et al.* (1929) and they reported that it did not prevent the occurrence of estrus, ovulation, fertilization or migration and implantation of embryos. However, in most instances resorption of the fetuses occurred. In contrast to these results were those of Peterson and co-workers (1952) who found no impairment in the reproductive performance of guinea pigs in which hyperthyroidism was produced by thyroxine administration. Hurst and Turner (1948) induced hyperthyroidism in female mice by thyroxine injections and found this to be detrimental to fertility which was manifested both by a low rate of conception and the birth of stillborn litters. Incomplete inhibition of ovarian activity was produced in female rabbits fed large doses of dessicated thyroid in an investigation by Chu and You (1944). According to Van Horn (1933) at least three rat units of estrone were required to produce artificial estrus in hyperthyroid castrate female rats. The medical literature is replete with reports on the effects both of the hyperthyroid and the hypothyroid state upon reproductive processes in the human female. It appears from the reports cited, that a wide species variation exists in respect to the effects of high levels of the thyroid hormone on reproductive processes.

As in the case of hyperthyroidism a deficiency in the thyroid hormone is reflected in many species by significant decreases from the normal in a number of reproductive functions. One aspect of the problem related to the thyroid hormone and embryonic mortality is the activity of the fetal thyroid gland. In sheep, for example, Gorbman *et al.* (1957) observed a hundred fold increase in the

iodine-concentrating ability of the fetal thyroid gland during early pregnancy. During the period when its iodine concentrating ability was still weak, the thyroid displayed a limited ability to form iodotyrosine, triidothyronine and thyroxine. Chapman *et al.* (1948) indicated that the human fetal thyroid begins to store iodine toward the end of the first trimester. These observations suggest that during the early phases of the gestation period the developing embryos are dependent upon the maternal thyroid for their thyroid hormone requirements.

A direct action of the thyroid gland upon ovarian function is indicated by the results of numerous investigators. Hypothyroidism, especially, appears to have a marked influence as attested by the work of Hammett (1926), Peterson *et al.* (1952) and others. Conversely, increased ovarian activity, as measured by numbers of developing Graafian follicles and primordial ova was demonstrated in female cretin rabbits by Kunde *et al.* (1929) following the feeding of desiccated thyroid material. However, a hormone level within an optimum range is a requisite for a normal ovarian response as shown by Chu and You (1944). Small doses of dried thyroid gland fed to thyroidectomized rabbits prevented the accompanying hyperthrophy of ovarian follicles whereas large doses had an inhibitory effect upon ovarian activity. There are a number of other publications describing a possible relationship between the thyroid hormone, the hormones of the ovaries and the pituitary gonadotropins. Among these reports are those of Soliman and Reineke (1954), Jones (1954), and Van Horn (1933).

The investigations on the effect of thyroid deficiency upon gestation have yielded contradictory results. For example, Krichesky (1939) found little or no effect on gestation in rabbits due to thyroidectomy performed from one and one-half hours to 12 days after mating. The results of Chu (1944) failed to support this contention. He stated that resorption and abortion of the embryos followed thyroidectomy in rabbits and that a thyroidectomy late in pregnancy resulted in the death of the fetuses prior to parturition. The results obtained by Krohn (1950) substantiate, in some aspects, those of the authors above.

Similar experiments on rats have resulted in contradictory results as shown by Nelson and Tobin (1937), Jones *et al.* (1946), and Krohn and White (1950). One result of special interest reported by several of the above investigators is that the young born of mothers treated with goitrogens all died by the fourth week after birth, suggesting that the goitrogens affected the thyroid glands of the young.

In mice the thyroid was destroyed by radioactive iodine and the results were reported by Gorbman (1950) and Bruce and Sloviter (1957). Again the results differ. Whereas Gorbman observed the production of only nine litters by more than 300 treated females during the first five post-treatment months, Bruce and Sloviter were unable to show any effect of the treatment upon fertility or litter frequency.

Since the precise role and effect of the hormone of the thyroid gland upon reproductive processes in the female are at present uncertain no definite con-

clusions can be derived from reports in the literature. For this reason, as stated elsewhere, the thyroid secretion rate was determined for all of the rats in our investigations to assure the administration of thyroxine at an optimum level during the experimental period.

Measurement of Thyroid Secretion Rate.

Many methods based on substitution therapy have been employed to ascertain the quantity of exogenous thyroid hormone necessary to return the thyroid deficient animal to normal. Among the techniques described in the literature are: (1) the goitrogenic techniques, and (2) the radioactive iodine techniques. The radioactive iodine technique was employed in the present investigation. The observation that exogenous thyroxine retarded thyroidal I^{131} uptake (Cornwall and Reineke, 1951) and release (Pipes *et al.*, 1950) provided the basis for indirectly determining thyroid secretion rate by an estimation of the amount of exogenous thyroxine necessary to suppress release of I^{131} from the thyroid gland. The method we employed was essentially that of Pipes *et al.* (1950) with minor modifications suggested by Pipes and others in the Department of Dairy Husbandry, Missouri Agricultural Experiment Station.

Deoxyribonucleic Acid (DNA) Measurements.

The concept that the DNA level in a tissue could be utilized as an index of true growth (an increase in cell number) in a tissue was first suggested by Boivin *et al.* (1948). In essence, their theory stated that whereas the mean DNA concentration may vary widely from one species to another, the amount of DNA in the nuclei of somatic cells of the different tissues of animals within a species is identical or is a constant. In the nuclei of spermatozoa and ova, however, the DNA in these haploid cells is one-half that of the diploid somatic cell nuclei of animals within a particular species. These observations have been confirmed by Davidson and Leslie (1950), Mirsky and Ris (1951), Thomson *et al.* (1953) and England and Mayer (1957).

The very narrow range of variability in the DNA concentration between somatic cell nuclei within a species has also been confirmed by spectrophotometric measurements despite differences in the size of the nucleus.

According to Davidson (1953), the stability of DNA content of the nucleus (designated as the "Boivin—Vendrely rule") has sometimes been interpreted as implying a physical constancy in the amount of DNA in every non-dividing nucleus. Chemical methods can yield only a mean value for the general population of nuclei under examination. Cytochemical methods show the distribution among individual cells within that population. However, both methods indicate that variations in DNA content fall within limits which are sufficiently narrow to make the DNA the least variable of all cell components. Pollister *et al.* (1951), for example, stated that while the actual DNA content of a population of nuclei may range as much as 15 percent above and below the mean, as determined spectrophotometrically, this variation is slight in comparison to the

difference of 2000 percent in the protein content of the nuclei of immature erythrocytes and of nerve cells in the same species.

The methods for expressing the DNA quantities in growing tissues are numerous depending upon the tissue under observation and the accuracy with which a reference standard can be established.

Influence of Vitamin B_{12} on Reproduction.

Vitamin B_{12} is an essential nutritional factor available in minute amounts from foods of animal origin. This vitamin has been shown as an especially important factor in the blood-formation process. The literature also implicates this vitamin with the growth and reproductive processes. In this review only a few of the more pertinent publications on vitamin B_{12} and its influence on reproductive physiology will be mentioned.

In the first reports, rats were employed as the experimental animals. When female rats were severely depleted of vitamin B_{12}, a high proportion of the offspring were afflicted with abnormalities according to O'Dell *et al.* (1951). Hydrocephalus was the principal anomaly observed, but defects of the eyes and bones were also common. The nature of the hydrocephalus has been described by Overholser *et al.* (1954) and Newberne and O'Dell (1958). Recently, studies were made on the central nervous system including observations on the peripheral nerves, lungs, kidneys and adrenal glands from vitamin B_{12} deficient and from control embryos by Newberne and O'Dell (1959). They found that a high percentage of the offspring of vitamin B_{12}-deficient rats were hydrocephalic at birth. The neurons and glial cells of the hydrocephalic brains showed irreversible degenerative changes.

In a series of experiments with swine begun in 1948, Heidebrecht *et al.* (1949) showed the necessity of vitamin B_{12} for maximum reproductive efficiency in these animals. Although gilts and sows fed basal rations composed of yellow corn, soybean meal, alfalfa meal and minerals reared from 70 to more than 80 percent of their pigs, the weaning weights of the young were suboptimal. The addition of various supplements including 10 percent alfalfa meal, iodine and vitamin A, or the injection or liver extract, did not improve reproduction or lactation. When the sows were fed basal rations fortified either with an APF supplement (containing vitamin B_{12}) or fish solubles, they reared more pigs than those on the control ration and the pigs were heavier at weaning. The inclusion of an APF supplement containing Aureomycin also produced significant increases in the average percent of pigs weaned and the average weaning weight per pig.

In a more practical type of investigation. Stevenson *et al.* (1954) fed pregnant sows a basal ration of yellow corn, oats, tankage, soybean meal, linseed meal, alfalfa meal and minerals. The basal ration was not improved, as measured by reproductive performance, by supplementation with riboflavin, pantothenic acid and condensed fish solubles. A vitamin B_{12}-Aureomycin supplement did influence lactation which resulted in an increased weaning weight of the offspring.

Hence, vitamin B_{12}, on the basis of these reports, may be an essential factor for reproduction and lactation. However, we must await the results of future research before definite conclusions regarding the exact role of this vitamin in reproductive physiology can be made. As the results of the present investigation with rats indicate, more knowledge is also needed regarding the vitamin B_{12} levels in the control or basal rations and the daily requirements of the female during gestation.

MATERIALS AND METHODS

Conception of the Investigational Plan

This investigation is a part of the cooperative projects in the field of animal reproduction in the Departments of Animal Husbandry and Agricultural Chemistry. The concept of the problem, presented in this publication was the result of a series of investigations in laboratory and farm animals previously conducted by members of these departments.

Experimental Animals

Rats were employed as experimental animals primarily because they were one of the species best suited for a pilot study such as this and had been employed in some of the previous studies in this laboratory. CNF virgin female rats 100 days old (Carworth-Nelson Farm rats, New York City, Rockland County, N.Y.) were used in this study. This breed has been used in a number of investigations in reproductive physiology.

During 1958-1959, forty-five female rats and five males were used. The averages body weight was 172 grams with a range from 160 to 210 grams. During 1959-1960, fifty-four female rats and 10 males were used, with an average body weight of 179 grams and a range of 162-220 grams.

Breeding of the Females

One of the earliest signs of estrus in the CNF female albino rat is ear quivering elicited by stroking her gently on the head or back. This reaction does not occur unless the rat is in estrus. As a test for estrus, vaginal smears were made with a small cotton swab on a tooth pick. The cotton was moistened with saline solution in order to perform the vaginal smears with the least amount of irritation. Cells on the cotton swab were smeared on a slide and stained with Wright's stain. In females in estrus, the smear contains cornified non-nucleated epithileal cells. If the smear diagnosis was positive the females were allowed to run with the males so that mating would take place at the normal time in the afternoon. Early the next morning the females were again examined and the presence of spermatozoa in the vagina was accepted as a sign of successful mating. The females showing a positive mating test were placed in separate cages.

Experimental Procedure

The first phase of this investigation was a preliminary trial made during 1958-1959 designed: (1) to assess the beneficial or harmful consequences of hormonal therapy during gestation and (2) to determine the therapeutic dosage and the ratio of progesterone to estrone necessary to produce a beneficial response in the intra-uterine environment.

During 1959-1960, the experiments were designed to explore the possibility of insuring a better intra-uterine environment and thus prevent prenatal loss by means of exogenous progesterone and estrogen plus the effect of the thyroid gland (Thyroxine) and vitamin B_{12}.

Hormone Administration

The hormones were injected subcutaneously in the abdominal region with the rat held in the left hand with the palm of the hand over the animal's back and the thumb and index finger folding the forelegs across the neck. The needle was inserted with a quick thrusting motion just above Poupart's ligament and the point pushed upward about an inch in the direction of the head. A preliminary trial had been made to determine the time of implantation in ten of the CNF rats. Implantation occurred about 83 hours after breeding; therefore, the hormones were injected at the fourth day of pregnancy to be sure that implantation had taken place. [The sex hormones and vitamin B_{12} were obtained from Nutritional Biochemicals Corporation (Ohio)].

Treated animals received the sex hormones dissolved in sesame oil, while control animals received an equivalent volume of the sesame oil only.

Hormone Preparation

Sesame oil was used as a vehicle for the administration of estradiol benzoate and progesterone. A homogeneous suspension was obtained by mixing the prescribed amount of the estrogen with 90 percent ethyl alcohol and then combining this mixture with sesame oil. The alcohol was removed by evaporation over a steam bath, after which the estrogenic compound was distributed throughout the sesame oil. The same technique was employed in the addition of progesterone to the oil suspension.

Following are the exact steps used in preparing the hormone preparations:

During the year of 1958-1959. Ratio of estradiol to progesterone, 1:2000.

1. Dissolve 50 mg. estradiol in 400 ml. of sesame oil.
2. Transfer 4 ml. from the above (containing .5 mg. estradiol) to the 250 ml. volumetric flask.
3. Add sesame oil to the 250 ml. mark.
4. Add 1 gram of progesterone to the 250 ml. volumetric flask.

The dosage per animal was ½ ml. of the above preparation (containing 1 mcg. estradiol plus 2 mg. progesterone; a hormone ratio of 1:2000).

Ratio of Estradiol to Progesterone, 1:1000.

1. Dissolve 50 mg. of estradiol in 200 ml. of sesame oil.
2. Transfer 4 ml. from the above solution (containing 1.0 mg. estradiol) to a 250 ml. volumetric flask.
3. Add sesame oil to 250 ml. mark.
4. Add one gram of progesterone to the 250 ml. volumetric flask.

The dosage per animal was ½ ml. of the above (containing 2 mcg. estradiol plus 2 mg. progesterone, ratio 1:1000).

During the year of 1959-1960. Ratio of Estradiol to Progesterone, 1:2000.

1. Dissolve 50 mg. estradiol in 200 ml. of sesame oil.
2. Transfer 8 ml. from the above solution to a 200 ml. volumetric flask.
3. Add sesame oil to 200 ml. mark.
4. Add 4 grams of progesterone to the 200 ml. volumetric flask.

The dosage used was 1/10 ml. per 100 grams body weight, each dose 1 mcg. estradiol plus 2 mg. progesterone.

Counting the Corpora Lutea

A number of preliminary experimental trials were necessary for the development of a satisfactory procedure for counting the corpora lutea. The first method did not give accurate results. In this method fatty tissues were removed from the corpora lutea. Then corpora were placed between two slides which were pressed with the fingers until the follicles burst. A count of the corpora lutea was then made under a binocular microscope.

In the second method a staining technique was employed. After removing the fat which surrounded the corpora lutea, the tissues were frozen during a ten-hour period, then fixed in a solution of Sudan Black for 4 to 8 hours. (Sudan Black, ½ percent in 70 percent alcohol, boiled for 10 minutes under a reflux condenser). In this method the object was to stain the corpora lutea without staining the follicular tissue. However, the results were not satisfactory.

The technique which we finally accepted as the most satisfactory was as follows:

1. Prepare a solution of 70 percent gum arabic and 30 percent gelatin and then add sufficient thymol blue to color this solution.
2. Separate the corpus luteum tissue from the fat surrounding it.
3. Place the tissue under a dissecting microscope.
4. Every corpus luteum was counted, then marked with the colored solution described in (1) above to prevent recounting of those previously tabulated.

X-ray studies of rat uteri were also made in an effort to study the reabsorption of embryos during different periods of gestation, especially on the 15th, 17th and 20th days of gestation. Since good X-ray photographs of the embryos depend upon the osseous tissue present and the development of this tissue was not sufficiently complete at some stages of pregnancy to give satisfactory photographs, this method was not used in the subsequent experiments.

Determination of the L-thyroxine Secretion Rate by I^{131} Technique

The method used was that described by Pipes *et al.* (1950). The thyroxine secretion rate of an animal is controlled by a hormone secreted by the anterior lobe of the pituitary gland, thyrotropin, which influences the secretion of thyroxine by the thyroid gland. When thyrotropin is secreted in increased amounts it stimulates the thyroid to increased thyroxine production. The secreted thyroxine in turn acts upon the pituitary to depress the secretion of more thyrotropin. Thus, the variation in thyroxine secretion is dependent upon the interplay between the concentration of these two hormones.

In the assay of thyroid secretion rate L-thyroxine was injected daily in increasing amounts. When the amount of injected thyroxine equalled the daily thyroxine secretion rate of the test rat, it blocked the discharge of thyrotropic hormone from the pituitary. In the absence of circulating thyrotropin, the discharge of thyroidal I^{131} ceases and the decline in radioactivity of the thyroid gland is prevented. The point on the curve where the decline is stopped is considered the L-thyroxine secretion rate equivalent of the rat under assay.

Radioactive Iodine

I^{131} was made available by the Oak Ridge National Laboratories, Oak Ridge, Tenn. Carrier-free sodium radioiodine (NaI^{131}) was diluted to the desired volume with physiological saline. The dose used was 2 millicuries of I^{131} per animal.

Thyroxine Solutions

Crystalline L-thyroxine from Travenol Laboratories was used. The purity of the sample was 99.1 percent. The required amount of crystalline thyroxine was dissolved in a minimum quantity of 0.1 sodium hydroxide solution and then diluted with distilled water to a volume near the required volume. Then 0.1 N hydrochloric acid was added until precipitation was just perceptible. Finally, a 0.1 N sodium hydroxide solution was added, drop by drop, until the solution became clear. The resultant solution was diluted to the required volume prior to its utilization. Thyroxine injections were made daily in quantities proportional to the body weight of the rat during the secretion rate determinations. All injections were made subcutaneously.

The CNF rats were housed at a uniform temperature of 78 ± 1° F. in a room artificially illuminated during normal daylight hours. They were given Purina Laboratory Chow and fresh water daily. Each rat was injected with 2 millicuries of carrier-free I^{131}. Forty-eight hours were allowed for the I^{131} fixation by the thyroid. External neck counts for thyroidal radioactivity were taken at this time and every 48 hours thereafter. Prior to making the radioactive counts each animal was anesthetized with ether, then placed on a lead plate with the neck resting on a scintillation probe containing a 2″ NaI crystal (Fig. 1). Care was taken in the placement of the animal to insure the same geometrical re-

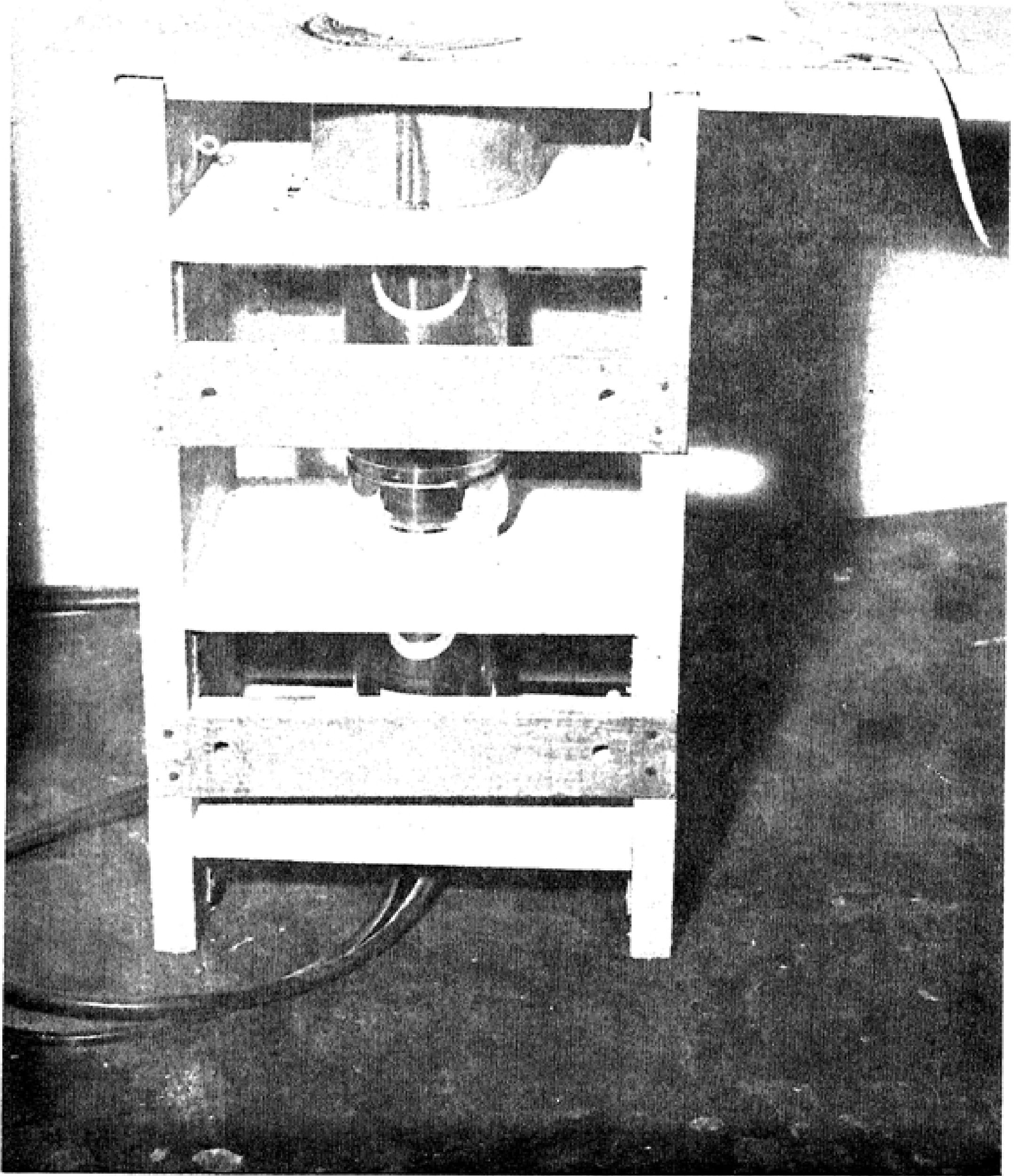

Figure 1 Anesthetized rat placed on a lead plate with neck resting on a scintillation probe.

lationship at each successive counting period. Thyroidal radioactivity was measured with a scintillation counter, Nuclear-Chicago (N.C.) Model D55, connected to a rate meter, N.C. Model 1620A. Conventional corrections were made for decay of isotope and background. Each rat was injected subcutaneously with .25 mcg/100 grams of body weight of L-thyroxine for 2 consecutive days beginning after the first I^{131} count. The thyroxine dose was increased to .5 mcg/100 grams of body weight daily for 2 consecutive days, with neck counts made on the day of each increase.

The other doses of L-thyroxine were .1 and .15 mcg/100 grams of body weight.

Thyroxine secretion rate (TSR) was determined by plotting the percent previous count with thyroxine dose. The dose which prevented further thyroidal I^{131} output in each rat (95-100 percent of the previous count) was estimated as TSR.

DNA Determinations

DNA was determined by the Webb and Levy method (1955).

Principle: DNA hydrolizes in TCA, trichloroacetic acid, and reacts with p-nitrophenylhydrazine when it is separated from interfering substances. The colored complex formed is determined colormetrically in alkaline solution. Color development obeys Beer's law, over a range of 10 to 300 ug of DNA.

P-nitrophenylhydrazine is specific for DNA and it has greater sensitivity than diphenylamine, which is also used for DNA determinations. Results are reproducible over a long range.

Procedure:

A. Extraction

1. Weigh 25 mg. samples of dry, fat-free uterine tissue.
2. Add 5 ml. of 5 percent TCA to each sample.
3. Cover lightly and place in boiling H_2O bath for 20 minutes.
4. Cool in cold H_2O bath.
5. Centrifuge 20 minutes.
6. Decant—*save supernatant.*
7. Re-extract precipitate (Using steps #2 through #6).
8. Combine supernatant from extractions #1 and #2. Dilute to vol. of 15 ml. with 5 percent TCA.
9. Extract may be stored in refrigerator overnight, if necessary, without harming the color-development procedure.

B. Color Development

1. Dilutions:

	P-nitrophenyl-hydrazine	5 Percent TCA	Final Volume
Blank		4 ml.	4.0 ml.
2 ml. standard	.2 ml.	2 ml.	4.2 ml.
2 ml. extract (unknown)	.2 ml.	2 ml.	4.2 ml.

2. Cover lightly and place in boiling H_2O bath for 20 minutes.
3. Cool in cold H_2O bath.
4. Add 5 to 10 ml. n-butylacetate.
5. Stopper and shake well.
6. Centrifuge, decant.
7. Transfer, quantitatively, 3 ml. aliquots to 5 ml. volumetric flasks.
8. Add 1 ml. of 2 N NaOH to each flask.
9. Dilute to volume with distilled H_2O.
10. Compare with distilled H_2O blank in a Beckman colorimeter.

Procedure for Purification of DNA

Reagents:

A. 0.1 N NaOH solution.
Add 4 grams of NaOH pellets to one liter (1000 ml) of distilled water.

B. 10:1 chloroform:octyl alcohol.
500 ml. chloroform plus 50 ml. octyl alcohol

Steps:

1. Dissolve crude yeast DNA in enough 0.1 N NaOH solution to permit solution. Add strong (10-15 percent) TCA until precipitate forms. Centrifuge, discard supernatant.
2. Redissolve precipitate in 0.1 N NaOH. Repeat step #1.
3. Redissolve precipitate in 0.1 N NaOH, and shake with an equal volume of the chloroform-octyl alcohol solution (Reagent B, above) in a separatory funnel. Protein separates out as a chloroform gel at the interface. This is done in the cold to prevent inactivation of the protein.
4. Pour bottom layer of mixture into a beaker, mix with chloroform-octyl alcohol, shake and again pour bottom or DNA layer into another beaker, repeat several times.
5. Precipitate DNA from bottom layer with strong TCA. Dry and grind in mortar and pestle.

RESULTS AND DISCUSSION

The main objectives of this study were (1) to determine the ovulation rate and embryonic death loss in CNF rats, (2) to determine the influence of certain hormones on these components of fertility and (3) to determine the degree of relationship between the thyroxine secretion rate of rats and their reproductive performance.

A total of 60 rats was used in preliminary experiments to refine the techniques of counting the number of corpora lutea and implanted embryos and to learn the method of determining the DNA content of the uterine tissue. Data from these rats were not included in the results. After the preliminary studies

two experiments were conducted dealing with the objectives listed above. The results will be presented separately for the two experiments.

EXPERIMENT I.

This experiment was conducted during 1958-1959. The plan for this experiment involving 45 rats is summarized in Table 1. Three groups of female rats were used with Group I serving as a control. Group II was injected with estradiol and progesterone at the ratio of 1:1000, whereas Group III received the same hormones except the ratio of estradiol to progesterone was 1:2000. Fifteen rats were included in each experimental group.

TABLE 1-TREATMENTS GIVEN FEMALE RATS IN THE FIRST EXPERIMENT CONDUCTED DURING 1958-1959

Group*	Treatment
I	Control
II	Treated with 2 mcg. of estradiol and 2 mg. of progesterone (1:1000) per animal for five days starting at the fourth day of pregnancy.
III	Treated with 1 mcg. of estradiol and 2 mg. of progesterone (1:2000) per animal for five days starting at the fourth day of pregnancy.

*All rats were sacrificed on the 15th day of gestation.

Ovulation Rate

Table 2 gives the numbers of corpora lutea found on the ovaries of the female rats of the three different groups. The number of corpora lutea was iden-

TABLE 2-AVERAGE NUMBER OF CORPORA LUTEA, LITTER SIZE AND NUMBER OF MISSING EMBRYOS AT THE 15TH DAY OF PREGNANCY IN FEMALE RATS IN EXPERIMENT I

Group	Avg. No. of* Corpora Lutea	Litter** Size	Avg. No. of *** Missing Embryos
I	11.33	6.67	4.66
II	11.73	8.93	2.80
III	12.52	9.40	3.12

*Differences between group means were not significant.
**Differences between group means were significant ($P < .025$).
***Differences between group means were significant ($P < .005$).

tical with the number of ova released from the ovary and was employed in the calculation of the embryonic death loss. This assumption might introduce some error because it does not take into account those ova which may not have left the follicle at the time of ovulation or those which produced identical twins. However, this error should not be of significant magnitude.

The number of corpora lutea per female varied from 11.33 in the control group to 12.52 in those females of Group III which received the hormones in the ratio of 1:2000. This difference between groups was not significant and could not have been affected by the hormone treatment since ovulation preceded treatment which was begun on the fourth day following mating.

Litter Size at the 15th Day of Pregnancy

The average number of embryos per female in the different groups is also shown in Table 2. An analysis of variance showed a significant difference ($P<.025$) between the group means. Female rats receiving injections of estradiol and progesterone at the ratio of 1:1000 during gestation possessed an average of 2.26 more embryos at 15 days of gestation than did the females in the control group. This was a significant difference ($P<.05$). Females in Group III which received the hormones in the ratio of 1:2000 possessed an average of 2.73 more embryos that the control females. This difference was also significant ($P<.05$). Differences between the means of the two treated groups were not statistically significant, however.

TABLE 3-DIFFERENCES BETWEEN AVERAGE LITTER SIZE IN CONTROL AND TREATED FEMALES NECESSARY FOR SIGNIFICANCE AT THE 5 PERCENT LEVEL OF PROBABILITY

Group	Average Litter Size	Litter Size as Compared to: Group I	Group II
III	9.40	2.73*	$0.47^{n.s.}$
II	8.93	2.26*	
I	6.67		

*Least significant difference ($P<.05$) between means is 2.25.

Embryonic Death Loss

The average number of ova not represented by embryos at 15 days of gestation for the three groups of female rats is shown in Table 2. The embryonic death loss was much less in the treated than in the control females. The analysis of variance showed that the three means did differ significantly. The test for least significant difference between means (Table 4) shows that the embryonic death losses were significantly lower ($P<.05$) in the treated females, although there was not a significant difference between the two groups of females which received the hormone treatment.

TABLE 4-DIFFERENCES IN EMBRYONIC DEATH LOSSES IN CONTROL AND TREATED FEMALES NECESSARY FOR SIGNIFICANCE AT THE 5 PERCENT LEVEL OF PROBABILITY

Group	Avg. Embryonic Death Loss	Avg. Loss as Compared to: Group III	Group II
I	4.60	1.87*	1.33*
II	3.27	0.54	
III	2.73		

*Least significant difference ($P< .05$) between means is 1.14.

Figure 2 illustrates more clearly the relationship between the treatment given the females and the amount of embryonic death loss by the 15th day of pregnancy. There is little doubt that the treatment with the estradiol-progesterone mixture in this study reduced the amount of embryonic death loss.

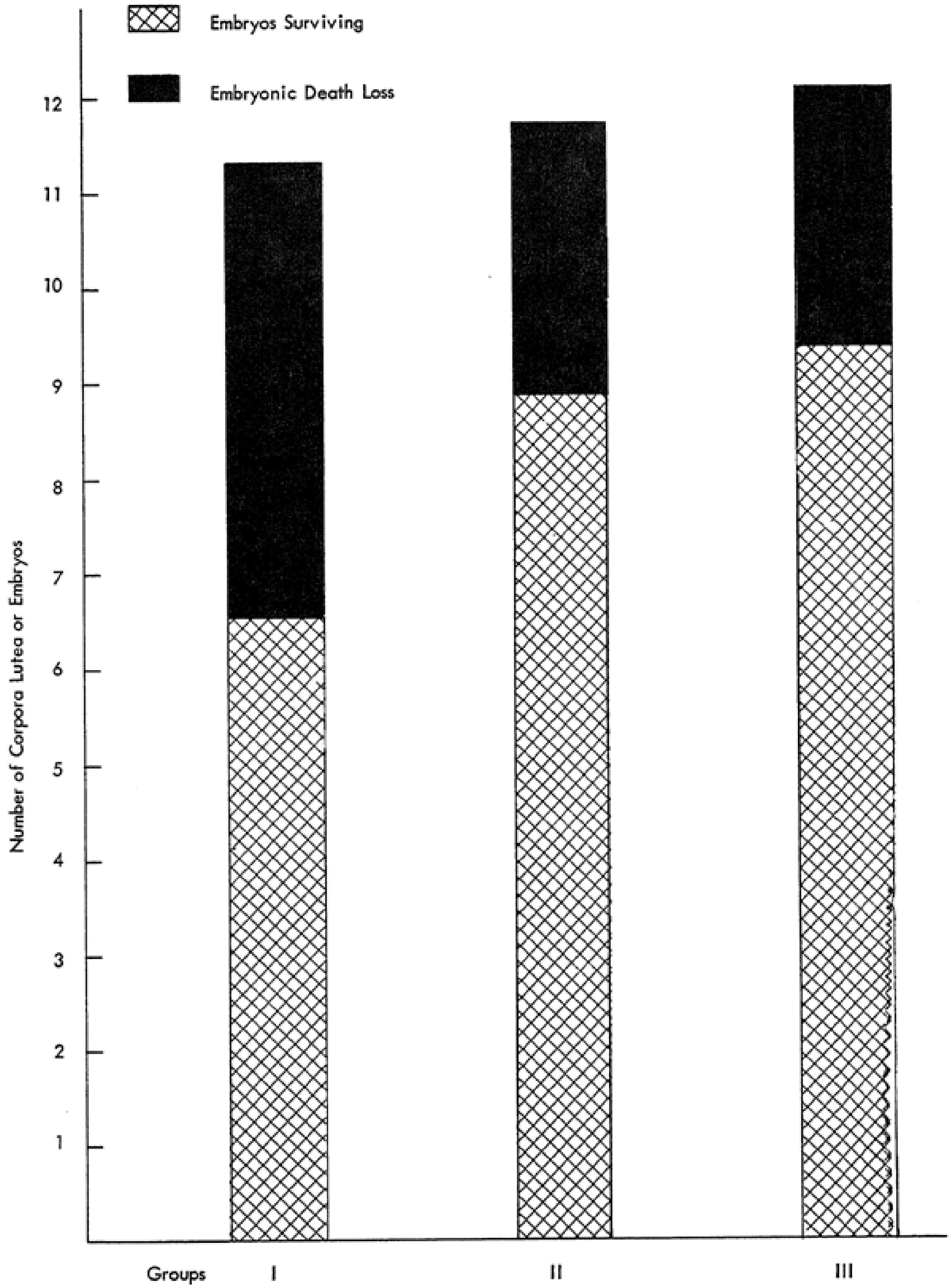

Figure 2 Showing Embryonic Death Loss in Female Rats as Influenced by Estrogen Progesterone Treatment During Pregnancy.

DNA Content of Uterine Tissue

The observation that treatment with the hormones reduced embryonic death losses in female rats suggested that this could have been due to a more favorable environment induced by the hormone treatment. Therefore, it was desirable to obtain some measurement of uterine growth. Uterine weights could give some indication of a more favorable environment, but a more accurate measure would be the amount of DNA in a given amount of uterine tissue. Since the DNA is constant within each cell nucleus, an increased amount in the tissue would be an indication of an increase in cell numbers or true growth.

The DNA concentration was determined in the uteri of the 45 female rats of the three different groups. The analysis of variance between means for the three different groups was not significant (Table 5), although the difference approached significance ($P < .10$).

TABLE 5-DNA CONCENTRATION OF UTERINE TISSUE IN TREATED AND CONTROL RATS IN THE FIRST EXPERIMENT (1958-1959)

Group	Mcg. of DNA per Mg. of DFFT*
I	39.38
II	42.15
III	45.85

*Differences between means were not significant ($P < .10$).

EXPERIMENT II.

Because of the encouraging results of the first experiment conducted in 1958-1959, a second experiment was conducted in 1959-1960. In the second experiment, however, estradiol and progesterone were administered on the basis of 100 grams of body weight in the live rats rather than on a per animal basis as in the first experiment. The number of groups of rats was also increased to six in the second experiment to include one group which received vitamin B_{12} beginning the first day of pregnancy, one which received L-thyroxine and a third which received vitamin B_{12} and L-thyroxine in addition to estradiol and progesterone. A detailed description of the treatments for each of the six groups is in Table 6.

TABLE 6-TREATMENT OF CNF RATS DURING 1959-1960

Group*	
I	Control
II	Treated with 1 mcg. estradiol and 2 mg. progesterone per 100 grams of body weight beginning the fourth day of pregnancy and continuing for 5 days.
III	Treated with 1 mcg. of estradiol and 2 mg. of progesterone per 100 grams of body weight and 2.5 mcg. of L-thyroxine per 100 grams of body weight betwee the fourth and the 15th day of pregnancy.
IV	Each animal received 1 mcg. of vitamin B_{12} per 100 grams of body weight at the first day of pregnancy.
V	Each animal received 2.5 mcg. of L-thyroxine per 100 grams of body weight for the same period as the rats in group III.
VI	Received 1 mcg. of estradiol and 2 mg. progesterone, 2.5 mcg. thyroxine and 1 mcg. of vitamin B_{12} described for groups III, IV and V.

*All rats were sacrificed on the 15th day of pregnancy.

Ovulation Rate

Table 7 gives the ovulation rate of the rats from the six different experimental groups as determined from corpora lutea counts. The average number varied from a low of 9.55 in females in group IV to a high of 12.44 in females in group VI, but the analysis of variance between group means showed no significant difference.

TABLE 7-AVERAGE NUMBER OF CORPORA LUTEA, LITTER SIZE AND NUMBER OF MISSING EMBRYOS AT THE 15TH DAY OF PREGNANCY IN FEMALE RATS IN EXPERIMENT II

Group	Avg. No. of* Corpora Lutea	Litter** Size	Avg. No. of ** Missing Embryos
I	11.66	6.33	5.33
II	11.67	9.00	2.67
III	12.11	9.67	2.44
IV	9.55	5.44	4.11
V	11.78	7.22	4.56
VI	12.45	9.89	2.56

*Differences between group means were not significant ($P < .10$).
**Differences between group means were significant ($P < .005$).

Litter Size at 15th Day of Pregnancy

Table 7 gives litter sizes in the treated and non-treated females. Litter size ranged from a low of 5.44 in group IV which received vitamin B_{12} alone to a high of 9.89 in group VI which received estradiol and progesterone in the ratio of 1:2000 as well as L-thyroxine and vitamin B_{12}. The analysis of variance showed that the group means differed significantly ($P < .005$). A further analysis of the variance between means using the test for least significant difference (Snedecor, 1956) is presented in Table 8. Data in this table show that females in groups

TABLE 8-DIFFERENCES BETWEEN LITTER SIZE IN SIX GROUPS OF FEMALE RATS IN THE SECOND EXPERIMENT NECESSARY FOR SIGNIFICANCE AT THE 5 PERCENT LEVEL OF PROBABILITY

Group	Average Litter Size	Difference Between Means as Compared to:				
		Group IV	Group I	Group V	Group II	Group III
VI	9.89	4.45*	3.56*	2.67	0.89	0.22
III	9.67	4.23*	3.34*	2.47	0.67	
II	9.00	3.56*	2.67	1.78		
V	7.22	1.78	0.89			
I	6.33	0.89				
IV	5.44					

*Least significant difference between means is 3.35 embryos.

II, III and IV all had a significantly ($P < .05$) larger litter size at 15 days of pregnancy than did the females in group IV which received only vitamin B_{12}. In addition, females in groups III and VI also had significantly higher ($P < .05$) litter size than did females in the control group.

These results substantiate those obtained in Experiment I in which it was found that injections of estradiol and progesterone given for five days beginning

the fourth day of pregnancy increased litter size in females sacrificed on the 15th day of pregnancy.

Data obtained in the second experiment failed to show any favorable effects on litter size when vitamin B_{12}, L-thyroxine or both were administered to the female rats during pregnancy. The data did suggest, however, that L-thyroxine might be helpful in this respect although the increase in litter size in both groups when this hormone was administered was not large enough to be of statistical significance. This portion of the study warrants further attention using more groups of females and a wider range of dosages of the hormones.

Embryonic Death Loss

The degree of embryonic death loss for the six groups of female rats in the second experiment is given in Table 9. It varied from 2.44 embryos lost per litter in females in Group III to 5.33 in females in the control group receiving no hormonal treatment. The analysis of variance showed a highly significant treatment effect ($P < .005$) on embryonic death loss. A further analysis of the data to determine which group means differed significantly ($P < .05$) is presented in Table 9. This analysis showed that embryonic death loss was significantly less in females in Groups II, III and VI than in the control females.

TABLE 9-DIFFERENCES IN EMBRYONIC DEATH LOSSES IN SIX GROUPS OF FEMALE RATS IN THE SECOND EXPERIMENT NECESSARY FOR SIGNIFICANCE AT THE 5 PERCENT LEVEL OF PROBABILITY

Group	Average Embryonic Death Loss	Difference as Compared to: Group III	Group VI	Group II	Group IV	Group V
I	5.33	2.89*	2.77*	2.66*	1.22	0.77
V	4.56	2.12*	2.00	1.89	0.45	
IV	4.11	1.67	1.55	1.44		
II	2.67	0.23	0.11			
VI	2.56	0.12				
III	2.44					

*Least significant difference between means was 2.08.

This again shows, as was true in the first experiment, that estradiol and progesterone reduced embryonic death losses in female rats. Figure 3 illustrates more clearly the relationship between the treatment given the females and the amount of embryonic death loss by the 15th day of pregnancy. Vitamin B_{12} and L-thyroxine, however, at the dosages administered and under the conditions of this experiment did not reduce embryonic death losses.

DNA Content of Uterine Tissue

Uterine tissue was again analyzed as in the first experiment to determine the mcg. of DNA per mg. of dried fat-free-tissue (DFFT). The results are summarized in Table 10. The group means varied from a low of 37.91 mcg. of DNA per mg. of DFFT in the control Group I to a high of 51.89 in females in Group III. The analysis of variance showed a highly significant difference ($P < .01$).

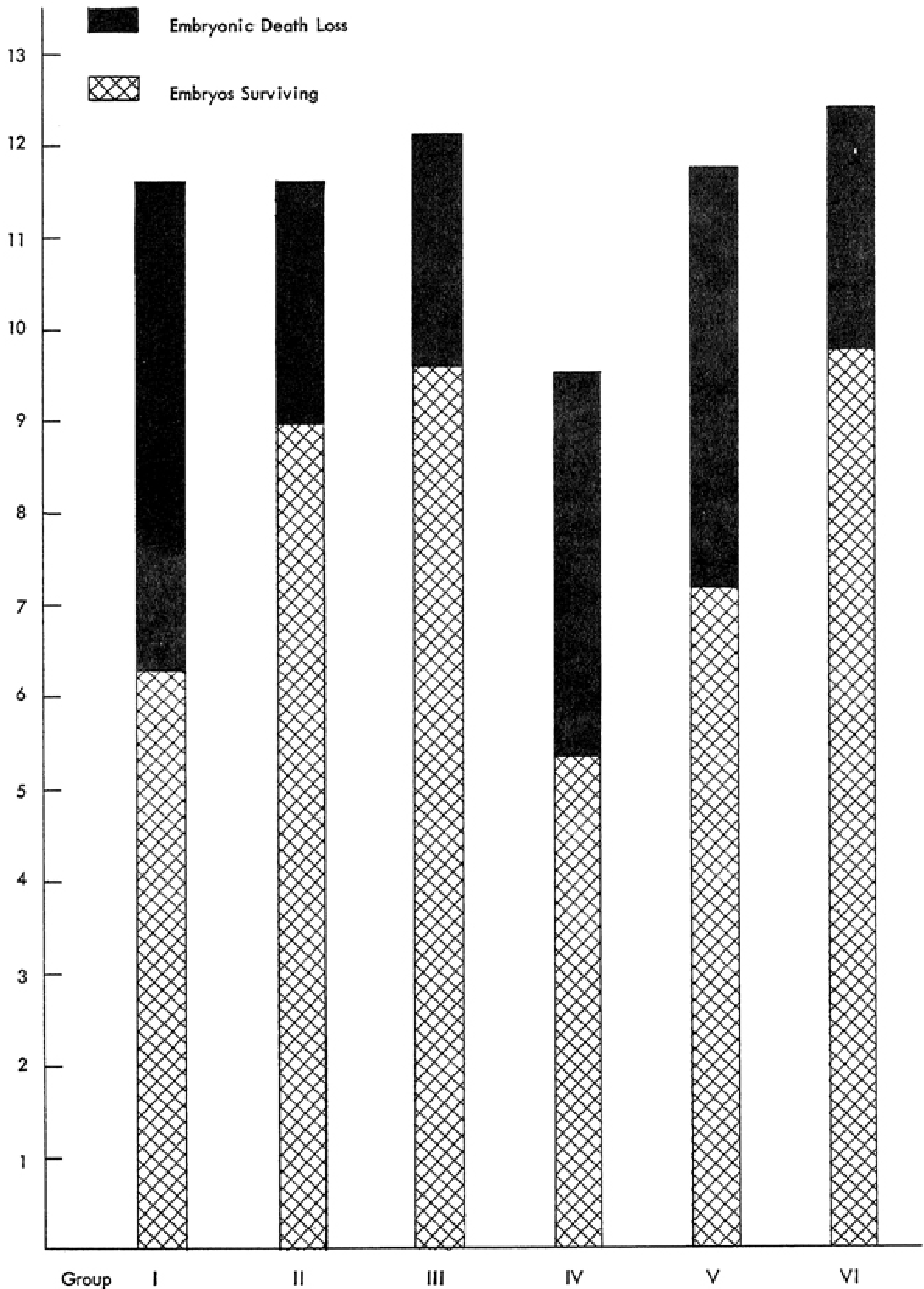

Figure 3 Showing Embryonic Death Loss in Female Rats as Influenced by Different Treatment (Second Experiment) During Pregnancy.

TABLE 10-DNA CONCENTRATION PER MG. OF UTERINE TISSUE IN TREATED AND NON-TREATED FEMALE RATS IN THE SECOND EXPERIMENT (1959-1960)

Group	Mcg. DNA per Mg. of DFFT
I	37.91
II	48.56
III	51.89
IV	41.77
V	45.12
VI	50.16

Table 11 shows that females in Groups II, III and VI had significantly more DNA per unit of uterine tissue than females in the control group. Since females in these three groups also had significantly less embryonic death losses than females in the control group, it is concluded that treatment with estradiol and progesterone increased true growth in the uterine tissue of the pregnant rat and thus provided a more favorable environment for the developing fetuses. These results are in agreement with those obtained with swine in this laboratory in which larger uteri, as determined by weight and linear measurements, were observed in the hormone-treated sows with low embryonic death loses (Reddy *et al.* 1958). However, in the sows DNA determinations were not made.

TABLE 11-DIFFERENCES IN DNA CONTENT OF UTERI OF TREATED AND NON-TREATED FEMALES NECESSARY FOR SIGNIFICANCE AT THE 5 PERCENT LEVEL OF PROBABILITY

Group	Avg. DNA per Mg. DFFT	Difference as Compared to Mean of: Group I	Group IV	Group V	Group II	Group VI
III	51.89	13.98*	10.12*	6.77	3.33	1.73
VI	50.16	12.25*	8.39	5.04	1.60	
II	48.56	10.65*	6.79	3.44		
V	45.12	7.21	3.35			
IV	41.77	3.86				
I	37.91					

*Least significant difference between means is 10.02.

Thyroid Secretion Rate as Related to Reproduction

The thyroid secretion rate (TSR) was measured in a total of 54 female rats when they weighed approximately 180 grams and just previous to placing them on experiment. The TSR in the rats tested ranged between 0.25 and 2.50 mcg. of L-thyroxine per 100 grams of body weight with an average of 1.00. Approximately one-third, or 18, of the rats had a TSR of 0.50 whereas another one-third, or 19, had a TSR of 1.00.

The data were analyzed to determine if ovulation rate varied with the TSR of the female rats in this study. Data grouped for this purpose are summarized in Table 12. Analysis of variance showed that the group means did not differ significantly. These results did indicate, however, that the ovulation rate tended to be higher in those females with the larger TSR. To determine if this relationship was real, the coefficient of correlation between these two variables was

TABLE 12-RELATIONSHIP BETWEEN THYROID SECRETION RATE AND OVULATION RATE, LITTER SIZE AND EMBRYONIC DEATH LOSS IN FEMALE RATS IN THE SECOND EXPERIMENT

TSR Rate	No. of Females	Avg. Ovul. Rate*	Litter Size*	Avg. Embryos Lost per Litter
0.25	3	11.33	8.00	3.33
0.50	18	10.78	7.44	3.33
1.00	19	11.63	7.53	4.05
1.50	7	12.00	7.86	4.14
2.00	5	12.60	9.60	3.00
2.50	2	13.50	11.50	2.00
Total	54	11.54	7.91	3.61

*None of the differences between means were significant.

calculated. It was found to be 0.219 and was not significant. These statistics show that there was no real relationship between TSR and ovulation rate among females used in this experiment.

The data were studied to determine if the TSR in the female rats was related to the number of embryos present in their reproductive tracts when they were sacrificed at the 15th day of pregnancy. Results of this phase of the investigation are summarized in Table 12. The analysis of variance again showed that the average litter size between females of the different groups did not differ significantly. A trend seemed to be evident for larger litters in females with the higher TSR, but the coefficient of correlation (.257) was not significant.

Since one-half of the females in the group of 54 did not receive added thyroxine during pregnancy, the correlation between litter size and TSR in treated and untreated groups was calculated. This was done because it was thought that additional thyroxine administered to female rats might result in detrimental effects on litter size in some of the females, especially those which normally had a high TSR.

The correlation between litter size and TSR in females not receiving thyroxine injections was positive but not significantly so ($r = .188$). Similar results were also obtained in the group of 27 female rats receiving thyroxine injections. In this group the correlation coefficient was also positive but not significant ($r = .278$).

The relationship between the TSR of female rats and the amount of embryonic death loss during pregnancy is also shown in Table 12. The analysis of variance again showed no significant differences between the means for the six groups of females. The coefficient of correlation was also low and negative ($r = -.052$).

The data from the 54 female rats were again divided into two groups; data from those receiving added thyroxine and data from the group of rats receiving no thyroxine. In the 24 females which did not receive thyroxine injections, the correlation between the TSR and number of embryos lost was positive and significant ($r = .393$, $P < .05$). This suggests that there was a higher embryonic death loss in females with the higher TSR in the untreated group.

In females which received injections of thyroxine, the coefficient of correlation between the TSR and embryonic death loss was negative and significant ($r = -.419, P < .05$). Therefore, the females with the highest TSR lost fewer embryos during pregnancy. These results seemed to be in disagreement with those obtained in females of the first group and are difficult to explain. They do suggest that there was some interaction between TSR and injected thyroxine that was favorable to the survival of the embryos. The physiological explanation of these observations is not known and should be investigated more fully in experiments specifically designed to clarify these results.

A large portion of our knowledge of thyroid physiology has been derived from observations on the effects of hypo- or hyper-activity of the thyroid gland. Considerable evidence has been presented to indicate that the optimal levels of thyroid hormone required vary with the individual and with environmental conditions, age, productive ability and physiological activity. Therefore, it is of importance to determine the normal rate of thyroid hormone secretion in experimental animals if this hormone appears to be a factor influencing the physiological process under investigation. Prior to the present investigation little attention has been given to the influence of the thyroid hormone upon uterine physiology and biochemistry or to the influence this hormone may have upon embryonic survival.

Some investigators have expressed the opinion that the maximum thyroxine secretion rate observed in a group of animals is the optimum level for that particular group or species (Pipes *et al.*, 1950; Pipes *et al.*, 1957).

In Table 12, note that the thyroid secretion rate of the rats used in this investigation varied between 0.25 and 2.50 micrograms of L-thyroxine per 100 grams of body weight. What is the optimum secretion rate for the rat? It is difficult on the basis of these results to suggest an answer to this question. Perhaps each individual animal has a secretion rate which is optimum for it.

Despite the wide variation in the thyroid secretion rates observed in the experimental animals, the treatment with the estrogen-progesterone mixture was apparently the major factor affecting embryonic survival. In these rats, thyroxine appears to play a minor role in this respect. Moreover, the synergism of estrogen and progesterone has a great effect on uterine growth. The question is, how does the uterus grow?

The work of Lyons (1943), Warwick *et al.* (1943), Herrick (1953), Dawson (1954), and Day (1959) support results of this investigation which show that the synergism of exogenous estradiol and progesterone creates a more favorable intra-uterine environment for the fetus and decreases embryonic death rate. The uterus has also been observed to respond actively to estrogens and progesterone. Several workers have shown that numerous endometrial mitoses can be correlated with estrogen and progesterone administration. Many studies in laboratory rodents, monkeys, and the human female have demonstrated repeatedly that the proliferative uterine growth phase in the endometrium is markedly influenced by the estrogens. These studies have given rise to our understanding of

the development and growth of the uterus from prepuberal state to the adult condition. DNA estimation in this investigation, however, allows a better understanding of uterine growth.

The mode of action of estrogen and progesterone is not fully understood although some investigations indicate that these hormones increase alkaline phosphatase while others have shown that they increase acid phosphatase. These unrelated studies do not provide a better understanding of the histochemical relationships in the endometrium. Electron microscopical studies of the female reproductive tract will undoubtedly enhance our comprehension of the various physiological phases during pregnancy.

The question, whether it is a more effective procedure to administer the hormones on the basis of body weight or on the basis of age has been raised. Day (1959) based his dosage of hormones on body weight whereas Reddy (1958) injected the same dose to all treated gilts when they had reached a particular age. These investigations yielded similar results. In the present investigation, during the first experiment, 100 day old rats were injected with 1 mcg. of estradiol and 2 mg. of progesterone (1:2000) per animal regardless of weight. These rats had an average weight of 172 grams (range of 160 to 210 grams).

In the second experiment, 100 day old rats with an average body weight of 179 grams (range from 162 to 240 grams) were injected with 1 mcg. of estradiol and 2 mg. of progesterone (1:2000) per 100 grams of body weight. The embryonic death rate during the first experiment was 22.5 percent whereas during the second it was 22.85 percent, suggesting that both methods of hormone administration yield similar results.

Moreover, the average DNA concentration in the uteri of the treated rats of the first and second trials, respectively, was 45.85 and 48.55 mcg. per mg. of DFFT. These figures indicate that there is no difference in the effects of the two methods of administration.

Additional vitamin B_{12} above that provided in the diet had no effect on uterine growth. It has been shown that a deficiency of vitamin B_{12} causes an abnormality in the reproductive physiology of the animal [O'Dell *et al.*, (1951); Newberne and O'Dell, 1959]. Therefore, it would appear that only a certain minimum amount of vitamin B_{12} is necessary to prevent reproductive abnormalities and quantities above this minimal level are of no benefit.

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