I The Effects of Feeding Diethylstilbestrol and a Forage Antiestrogen on the Reproduction of Female Mink. (Mustela vison).

II The Effects of Various Protein and Energy Levels on the Maintenance and Early Growth of Mink. (Mustela vison).

by

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We accept this thesis as conforming to the required standard.

THE UNIVERSITY OF BRITISH COLUMBIA

APRIL 1969
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ABSTRACT

In Part A of Study I, six groups of female standard mink were fed (once daily) either 5, 10, or 15 mcgm. of DES/mink/day or a forage antiestrogen extract at levels of 5, 10, or 15 gm. equivalent of alfalfa/mink/day. In Part B, seven groups of female pastel mink were fed (once daily) 15 mcgm. of DES/mink/day prior to and at various stages of the gestation period. Also, the antiestrogen extract was again fed (once daily) at levels of 5, 10, or 15 gm. equivalent of alfalfa to three groups of female pastel mink after implantation of the fertilized egg had occurred.

From the results of Part A and B of Study I it is evident that DES at levels as low as 5 to 15 mcgm/mink/day caused complete reproductive failure. At the 15 mcgm. level it is possible to prevent or disrupt pregnancy if the hormone is administered for at least one week during the period starting one week before breeding and ending 30 days prior to parturition. The forage antiestrogen at levels between 5 and 15 gm. equivalent of alfalfa reduced the number of kits per litter from 5.8 to 1.6. The feeding of various levels of DES or the antiestrogen extract did not adhere to a dose/response relationship either in interrupting pregnancy or reducing the number of kits per litter.
The DES, fed at levels of 5, 10, or 15 mcgm./mink/day was a more potent antifertility agent than the forage antiestrogen fed at levels equivalent to 5, 10, or 15 gm. of alfalfa.

In Part A of Study II, 200 mature male and female mink of a variety of colour phases were divided into five groups. The groups were fed (twice daily) rations varying in crude protein (36.6 to 44.8%, on a d.m. basis) and gross fat (21.3 to 28.9%, on a d.m. basis). In Part B, 280 pastel and standard kits (6-7 wks. old) were fed (twice daily) rations varying in crude protein (39.0 to 54.3%, on a d.m. basis) and gross energy (5.20 to 5.52 Kcal/gm. dry feed).

From the results of Part A and B of Study II it is evident that rations containing 35 to 40% crude protein and 20 to 25% fat, on a dry matter basis, are satisfactory for the late growth and maintenance requirements of mink. The mean apparent digestibility coefficients obtained in these studies for dry matter, protein, fat, and energy are 66.38, 74.05, 87.20, and 73.74%, respectively. As the apparent digestible energy (A.D.E.) was raised from 353.4 Kcal/100 gm. dry matter to 426.0 Kcal/100 gm. and as the apparent digestible nitrogen (A.D.N.) level reached 4.980 gm/100 gm
dry matter, the early growth of both male and female mink was improved. Maximum early growth rate was obtained in kits when the ration contained 85.54 Kcal of A.D.E./gm A.D.N. (13.6 Kcal gross energy/gm gross protein). Also, increasing A.D.E. content in the diets had a definite protein-sparing effect. Maximum nitrogen retention was obtained when the diet contained 426.0 Kcal of A.D.E./100 gm. of dry feed. Finally the results suggest that the growing male kits (from 6-28 weeks of age) were capable of utilizing feeds more efficiently than female kits of the same age.
ACKNOWLEDGEMENTS

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STUDY I

The Effects of Feeding Diethylstilbestrol and a Forage Antiestrogen on the Reproduction of Mink, (Mustela vison).

PART A. After Implantation of Ova.

PART B. Before Implantation of Ova.
I. Introduction

It has become evident that various hormones of animal and plant origin and certain synthetic hormones have a marked effect on fertility. Occasionally, in the past, the contamination of feeds by estrogens has caused reproductive disturbances resulting in a decrease in the number of litters in breeding colonies of laboratory animals. In commercial enterprises, the area which has been most drastically affected by estrogen contamination of feeds is the mink industry. The synthetic estrogen, diethylstilbestrol (DES), for a number of years, caused marked reductions in the annual kit production across Canada and the United States. During the late 1950's, the indiscriminate use of DES as a "growth stimulant" resulted in the introduction of DES into mink feeds via pellet-inserted chicken heads, poultry viscera or dried tankage, and beef tripe from animals implanted with or fed DES prior to slaughter.

Today, however, legislation at the turn of the decade has limited the use of DES in livestock feeding. As a result, less contamination of slaughterhouse by-products, especially in the poultry industry, has safeguarded against the major introductory routes of DES into mink rations. The drastic effects of estrogens on fertility, however, still causes concern that other possible routes of estrogen contamination exist.
II. Literature Review

A. Estrogens and Antiestrogens

Reproductive difficulties in animals grazing certain kinds of pastures were first noticed in Australia in connection with the increased use of subterranean clover (Trifolium subterraneum) as a sheep pasture. Since then, a number of estrogen-like compounds have been isolated from a variety of plants (95,96). The most significant plant estrogen is coumestrol isolated from ladino clover (Trifolium repens) by Bickoff, et al (14) in 1957. Kitts, et al (77,78) demonstrated that the level of estrogenic activity in certain legumes and grasses (e.g. alfalfa, ladino clover, and birdsfoot trefoil) was highest in spring and decreased toward autumn. Legg, et al (80) felt that the level of estrogenic activity may be associated in some way with the reproductive status of the plant material from which they were recovered.

In 1960, Bickoff, et al (15) found inhibitors to estrogen activity in some alfalfa samples. Adler (1) in 1962 demonstrated that as well as possessing estrogenic properties, alfalfa also contained antiestrogenic properties. Ostrovsky and Kitts (97) have suggested that birdsfoot trefoil contains an antiestrogen similar to
that found in alfalfa. Cook and Kitts\textsuperscript{33} have reported the presence of an antiestrogenic substance in yellow pine needles. In 1965, Adler\textsuperscript{2} established the presence of estrogen inhibitory activity in oat hay (Avena satina) and fahli clover hay (Trifolium alexandrinum var. Fahli).

Inhibition of fertility by estrogens and anti-estrogens can be achieved by interfering with normal mechanisms at any one of several vulnerable points in the reproductive process.\textsuperscript{114} These include impairment of gametogenesis by compounds having direct action on germ cells,\textsuperscript{72,112} prevention of ovulation by administering steroids which suppress pituitary production of gonadotrophins,\textsuperscript{111,13} interference with the estrogen/progesterone ratios essential for tubal transport and implantation of the fertilized ovum and destruction of ova and developing embryos with antimetabolites.

1) Suppression of Pituitary Gonadotrophins

The antifertility effects due to prevention of ovulation can be obtained by chemical antagonism to the secretion of gonadotrophins.\textsuperscript{72} Compounds which can antagonize the secretion of gonadotrophins include estrogens, such as DES,\textsuperscript{72} and norethynodrel;\textsuperscript{112} androgens, such as ethinyl androstenediol 3-cyclohexyl-lproprionate\textsuperscript{13}; and the progestational steroids.\textsuperscript{72}
The inhibition of the pituitary gonadotrophin secretion results in an impairment of follicular development and subsequent ovulation of the mature ovum.

2) The effects of estrogens and antiestrogens on transport of the fertilized ovum.

a) Estrogens

Following the initial work of Parkes and Bellerby, and Smith with mice and rats respectively and Kelly with guinea pigs, Burdick and Pincus in 1934 suggested that the daily injection of an estrogen (eotrin) into mice and rabbits during the preimplantation period resulted in the retention of ova in the Fallopian tubes. It was felt that the retention of the ova was probably due to either a closure of the tubo-uterine junction or an alteration in the nature of the ciliary movement which subsequently resulted in the inhibition of pregnancy. Also, Burdick and Pincus found that all ova, whether they descended into the uterus or remain in the tubes, showed definite signs of degeneration by the fourth day after copulation. In 1936, Whitney and Burdick showed that the injection of estrogenic substances during the preimplantation period
caused retention of ova in the Fallopian tubes and originated the term "tube-locking" of ova. Whitney and Burdick\textsuperscript{138} found that tube-locking occurred when low doses (5-10 R.U.\textsuperscript{1}) of an estrogenic substance was injected into the rat, but suggested that with heavier doses of estrogens increased ova transport occurred due to increased ciliary motility. It was later demonstrated by Burdick and Whitney\textsuperscript{23,24} that injections of 100 to 500 R.U. of an estrogenic substance accelerated the passage of fertilized ova through the Fallopian tubes of mice. Ova were frequently found in the uterus even in the two-celled stage, 30 to 40 hrs. after finding the vaginal plug. This is at least 30 hours sooner than usual. In 1938, Whitney and Burdick\textsuperscript{139} obtained similar results after injecting rabbits with a single massive dose (5000 R.U.) of an estrogenic substance. Synthetic estrogens, such as DES, administered in early pregnancy also accelerated the rate of tubal passage.\textsuperscript{22}

To investigate further the effects of estrogens on pregnancy, Dreisbach,\textsuperscript{44} in 1959, attempted to find the smallest dose of hormone and the most

1. R.U. = rat unit.
appropriate time of administration which would produce alterations in tubal transport in the rat. He found that as little as 0.02 mg/kg. body weight was effective during the period of tubal transport and the period most sensitive to estrogen administration was the third day post-coitum. Also in 1959, Greenwald\textsuperscript{58} studied the comparative effectiveness of estrogens in interrupting pregnancy in the rabbit. He found that estradiol benzoate was the most effective estrogen in interrupting pregnancy when compared with estrone and DES.

In 1961\textsuperscript{59} and again in 1963\textsuperscript{61} Greenwald, in his study of the transport of ova through the rabbit oviduct, reported results which were contrary to the tube-locking effect of estrogens in the rat reported by Whitney and Burdick\textsuperscript{21} in 1936 and the acceleration effect on fertilized ova reported by Burdick and Whitney in 1937. Greenwald\textsuperscript{59} reported that while the injection of 250 μg of estradiol resulted in the tube retention of ova, 25 μg of the same compound accelerated ova transport into the uterus; many of the ova were then expelled into the vagina. Also, Greenwald\textsuperscript{59} found that when the ova were retained in the tube by the injection of a large
dose of estrogen, the ova were blocked, not at the tubo-uterine junction as reported earlier, but at the beginning of the isthmus or ampullary-isthmus junction. Later in 1961, Greenwald found that after a single injection of estradiol cyclopentylpropionate, on the morning sperm were found in the vagina, tubal and uterine motility were increased so that the fertilized eggs were expelled from the uterus by 48 hrs. post-coitum. Deanesly, in 1963, concurrently found that low doses of estradiol benzoate, given immediately after mating caused premature expulsion of fertilized eggs from the Fallopian tubes in the guinea pig. He suggested that the term "tube-locking" should be dropped. In 1964, Banik and Pincus showed that as much as 20 μg/rat of estrone administered on Day 1 of pregnancy expells the eggs from the tube into the uterus in about 20-24 hours on Day 2. There was no evidence that a large quantity of estrone (250 μg/rat) blocks the fertilized egg at the utero-tubal junction. Also in 1964, Harper using autoradiographic techniques to trace the movement of "artificial eggs" (radioactive spheres), demonstrated that estradiol benzoate, in the dose administered (0.5 μg), had an accelerating effect on the passage of the spheres through the tube, and also
through the uterus. It was observed, however, that despite the accelerating effect of the estrogen a certain percentage of spheres were trapped at the junction of the ampulla and the isthmus. Recently, in 1968, Humphrey and Martin presented experiments in which one level of estradiol-17β (0.4 μg/day) caused both retention and loss of ova in mice. Retention did not only involve blockage at the utero-tubal junction, since ova were also located in the ampulla. Later in 1968, Humphrey demonstrated that sphincters at the ampulla-isthmus and isthmo-uterine (utero-tubal) junctions are capable of delaying fertilized ova during their sojourn through the Fallopian tubes.

b) **Antiestrogens**

Although not as extensively studied as the estrogens, both the natural and synthetic antiestrogens are capable of interfering with pregnancy by altering the rate of tubal transport of the fertilized ovum. It was first reported by Burdick, Emmerson and Whitney, in 1940 that testosterone propionate injected in adequate amounts into mice will cause the retention of blastocysts in the oviducts. The results showed that 2.0 mg. daily was the liminal amount of testosterone propionate necessary to cause retention of blastocysts in the tubes beyond 100 hrs. after the formation of the vaginal plug. In 1961, Greenwald with a single injection of progesterone caused some acceleration in ova transport. In only one instance were
ova recovered from the uterus at 48 hrs. after administration of 25 mg. of progesterone. Acceleration of ova was also obtained with the injection of similar amounts of progestin.59

In 1964, Harper,64 found that progesterone delayed movement of the radioactive spheres through the ampulla very markedly, but if the spheres reached the isthmus they passed into the uterus on all occasions. Chang30 in 1964 and Chang and Yanagimachi31 in 1965 showed that a number of antiestrogens interfered with ovum transport in rabbits, causing acceleration in some and retention in others. In 1968, Humphrey and Martin71 demonstrated that DMS1. (100µg/day), MER-252. (2.0 mg/day) and MRL-373. (2.0 mg/day) administered subcutaneously on Days 1, 2 and 3 post-coitum to female mice resulted in retention of ova in the oviduct. As well, the antiestrogens delayed the development of the retained ova and ova were prominently recovered from the tube in the morula rather than the blastula stage.

Although antiestrogens are capable of disrupting the passage of fertilized ova through the Fallopian tubes, they are even more effective as anti-implantation agents. In 1959, Dreisbach44 reported that pregnant rats were most

1. DMS = dimethylstilbesterol
2. MER-25 = 1-(p-2-diethylamino-ethoxyphenyl)-1-phenyl-2-anisyl ethanol
3. MRL-37 = corresponding ethane of MER-25
sensitive to antiestrogen administration thus differs from the time when estrogens produce their greatest effect, that is when the ova are in the Fallopian tubes.

c) **Mechanism of Action:**

1) **Estrogens**

   With regards to tube-locking, it is presently felt that the ampullary-isthmic junction rather than the utero-tubal junction is the primary block to egg transport in the rabbit. According to Wintenberger-Torrès the major block to egg transport in the ewe occurs at this junction. But Greenwald feels that the utero-tubal junction is probably the primary barrier in some species.

   It is important to comprehend the nature of the blocking mechanisms not only to understand how large doses of estrogenic compounds act to retain ova in the tube, but also to determine how low levels of estrogens prematurely advance the time of opening of the ampullary-isthmic junction. The mechanism of action may also explain why Whitney and Burdick found that low doses of an estrogen resulted in tube-locking and large doses caused ova acceleration; results which are contradictory to the more recent reports.

   The first theory proposed was by Hartman in 1939. Hartman suggested that the administration of an estrogen caused an alteration in the estrogen-progesterone balance,
which caused a variation in the rate and amplitude of muscle activity in the tube and in turn the rate of passage of the ova.

A second theory was proposed by Alden\textsuperscript{5} in 1942. From his observations by transillumination, Alden felt that while ciliary action was the primary force in effecting the entrance of the egg into and through the cephalic ampulla, action of the tubal musculature was the controlling factor governing further advancement. The administration of an estrogen, therefore, would result in the closure of the lumen sphincter at the site of the block due to the contraction of muscle layers.

Black and Asdell\textsuperscript{16,17} later proposed a third theory to account for the retention of the fertilized ova. They suggested that there was a slight or complete absence of activity of the circular muscle at the site of the blocked ova. Also movement induced by the circular muscle is sufficient to account for transport of ova through all of the oviduct except the isthmus portion immediately anterior to the tubo-uterine junction.\textsuperscript{16} The absence of circular muscle activity at the uterine end of the oviduct may partially account for ovum delay in this portion of the tube.\textsuperscript{17} Black and Asdell\textsuperscript{17} have attempted to demonstrate the presence of edema in the tubo-uterine junction, but
these attempts have failed. They feel, however, that the possibility still exists that it is present since the degree of edema would not have to be great to be effective in closing the tube.

Edgar and Asdell, in 1960, from their study of the valve-like action of the utero-tubal junction in the ewe, were able to propose a fourth theory. Evidence they presented indicates that estrogens cause edema on the subserosa and muscle coats of the wall of the utero-tubal junction of the ewe. It seems likely that the edema causes an increase in the degree of flexure of the tubal wall in the immediate area and the two phenomena contribute, along with the narrow lumen and the folding of the tubal lining to the valve-like action of the utero-tubal junction. In support of this theory, Voyes, et al found it increasingly difficult to force fluid from the ampulla through the utero-tubal junction as the dosage of estrogen was increased.

Finally a fifth theory was proposed by Wintenburger-Torres in 1961 which suggests that strong antiperistaltic contractions of the isthmus maintain the egg in the ampulla and prevent any further progression down the uterine tube.

At present there is little evidence to support any of these theories. It would appear, however, that the injection of estrogen interferes with progesterone dominated mechanisms. Greenwald in 1957 demonstrated that mucin, a mucopolysaccharide, secreted by the tubal epithelium onto the ova is inhibited by the administration of estradiol in rabbits. It is not known, however, whether or not the
reduced mucin layer plays a significant role in the retention of fertilized ova.

In conclusion ovum transport is aided by the contractile activity of the tube which is co-ordinated by anatomical and physiological mechanisms. Many of the mechanisms of reception and transport of ova are markedly affected by the complex actions and interactions between the ovarian steroids and pituitary hormones, the sympathetic nervous system and the adrenal steroids. Any abnormal alterations in hormone ratios, therefore, would cause alterations in both reception and transport of the fertilized ova.

ii) Antiestrogens

To determine the mechanisms by which antiestrogens interrupt pregnancy, it was first necessary to determine whether these compounds were acting either by virtue of their estrogenic or antiestrogenic properties. It was shown by Martin, et al. in 1960 that antiestrogens are also estrogenic (or pro-estrogenic) in high doses. Martin and his colleagues did, however, show that differences exist between the relative potency of dimethylstilbesterol, the most potent anti-strogen tested and estradiol as anti-fertility agents and their relative potency as estrogens. Also, differences existed between their capacities to interrupt pregnancy in single doses. It was therefore concluded that DMS, at least, probably acts by virtue of its
antiestrogenic properties. In the same year, Emmens, et al. found that when injected together, DMS did not antagonize the anti-fertility action of estradiol. This was regarded as very good evidence that DMS is, in fact, acting as a proestrogen in preventing fertility. In 1963, Martin, et al. demonstrated that a number of synthetic anti-estrogens interrupted early pregnancy in mice. It was still not clear, however, whether they did so by reason of their anti-estrogenic or proestrogenic activity since, in the series tested, both properties were highly correlated with the effectiveness of the compound in interrupting pregnancy. Again in 1964, Emmens, et al. while studying the estrogenic and antiestrogenic activity of compounds related to DES, found a high correlation between proestrogenic, anti-estrogenic and anti-fertility potencies in these compounds. It was still hazardous, however, from the evidence presented, to ascribe the anti-fertility action of antiestrogens to either their proestrogenic or antiestrogenic properties.

Up until this point the majority of the evidence presented was interpreted in favour of DMS acting as an anti-fertility agent by virtue of its antiestrogenic activity. However, in 1964, Stone and Emmers, found that sensitivity to single injections of both estradiol and DMS was highest approximately 72 hrs. after mating and had decreased after 96 hrs. In view of the similar action of the two compounds and the lack of consistent antagonism when
injected together, it was suggested that the anti-
fertility action of DMS in the mouse was due to its
pro-estrogenic activity. In a second fertility study,
Stone and Emmens found that a single, simultaneous
injection of DMS and estradiol to 60 hrs. pseudopregnant
rats showed only an additive effect in inhibiting deciduoma
formation and suggested that DMS was acting as a proestrogen
rather than an antiestrogen. In 1965, Emmens by
further detailed analysis of the activities of DMS by different
routes of administration in the rat and mouse, decided that in
the case of that particular compound the anti-fertility
effect is very probably due to its proestrogenic action.
However, among the non-steroids, some, including MRL-37
and MER-25 may have anti-fertility effects unrelated or
incompletely related to estrogenic activity and possibly
related to antiestrogenic activity. Finally, in 1968,
Humphrey and Martin presented results which confirm the
view that most antiestrogens are acting by virtue of their
proestrogenic activity.
The effects of estrogens and antiestrogens on implantation.

The ability of estrogens and anti-estrogens to interrupt pregnancy, administered after the ovum has normally entered the uterus, has been well described in the literature. In 1938, Parkes, et al.\textsuperscript{99} demonstrated that oral administration of estrogens is effective in preventing implantation of the blastocyst. DES, prepared by Dodds, Goldberg, Lawson and Robinson\textsuperscript{42}, the biological properties of which have been described by Dodds, Lawson and Noble,\textsuperscript{43} is reported highly active by mouth. Oral administration of DES is highly effective in preventing implantation in rats at levels as low as 5 to 16ug.\textsuperscript{99}

D'Amour, et al,\textsuperscript{36} in 1933, presented evidence indicating that appropriate dosages of estrin, administered during the early stages of gestation, prevented implantation in the rat and that this effect was not overcome by simultaneous injection of an estrogen antagonist, such as progesterone. In 1934, D'Amour and Gustavson\textsuperscript{37} suggested that the action of estrin might be a direct one, affecting the uterine mucosa in such a manner as to prevent implantation.

Greenwald,\textsuperscript{57} in 1957, has reported that the administration of estrogens in the rabbit 72 hrs. post-coitum involves an alteration in the progesterone dominated uterine environment prevents the implantation of ova. Segal and Nelson\textsuperscript{114} reported that the anti-estrogen MER-25 can also interfere with the progestational support of the endometrium, which results in the reduced development of implantation sites.
Dreisback\textsuperscript{31,44} found that as little as 0.1 mg/kg. body weight of estrone, injected subcutaneously, was necessary to prevent implantation in the rat when the ova were in the uterus. Fowler and Edwards,\textsuperscript{113} in 1960, found that mice, injected 7 days after mating with 1.0 µg. of estradiol benzoate or progesterone had no implanted embryos on the day of autopsy. Cochrane and Shackelford\textsuperscript{32} attempted to hasten ova implantation in mink by daily injections of a combination of 2.4 mg. of progesterone and 24 µg. of estradiol benzoate, beginning 7 to 22 days post-coitum. The hormonal treatment caused most females to be barren. In the animals which did produce kits, the administration of the exogenous estrogen alone or in combination with progesterone, resulted in reduced litter size and caused further delay of ovo-implantation.

The progestational proliferation or preparation of the endometrium, necessary for implantation of eggs, depends upon a delicate balance between estrogens and progesterone.\textsuperscript{9} It has been adequately reported that both exogenous estrogens,\textsuperscript{113} and anti-estrogens\textsuperscript{51,45} interfere with the formation of deciduoma prior to implantation of the fertilized egg.

At physiological levels, the estrogens are essential for implantation to occur. Nutting and Meyer,\textsuperscript{94} in 1964, reported that the optimum amount of estrone required for inducing implantation at the normal time appears to be between 0.3 and 1.0 µg. daily. If the level of estrone is below a threshold level a delay in nidation occurs, but if the estrone level is above the threshold level, nidation is induced.\textsuperscript{94} Progesterone appears to be vitally concerned with maintaining the viability of the blastocyst during the extended preimplantation period.\textsuperscript{88} Estrogens play an active part in determining whether implantation will occur,
but only if relatively large quantities of progesterone are available to ensure the presence of viable blastocysts. Humphrey, in 1967, Smith and Biggers and Smith, in 1968, have shown that in the absence of estrogens or progesterone implantation will not occur, but when a uterus is primed with progesterone, the injection of sufficient amounts of an estrogen (0.024 μg. estradiol, 0.5 μg. 17β-estradiol benzoate) will result in implantation of the fertilized ovum.

Mechanism of Action

In order to understand the mechanism by which estrogens and antiestrogens interfere with implantation, the suggested mechanism of ovum implantation must first be discussed. In 1952, Shelesnyak reported that antihistamines, such as diphenhydramine or ephedrine hydrochloride, inhibited the formation of deciduoma when introduced as a pellet or in solution into the uterine lumen. Later, Shelesnyak, in 1955, and Carlsen, et al., in 1961, also showed that antihistamines inhibited deciduoma formation, and that this inhibition was reversed by the concurrent administration of progesterone. These results suggested that antihistamines disturb the critical estrogen/progesterone balance for the induction and growth of the decidual cell reaction. In 1957, Shelesnyak postulated that histamine by some means was released from the blastocyst and that histamine, the deciduoma inducing factor, plus progesterone operating on a progravid uterus are required for successful decidua
development. In 1958, Johnson and Shelesnyak and Spaziani demonstrated that the histamine was not produced by the blastocyst, but was produced by the uterus and that the release of the uterine histamine is under estrogenic control. In 1959, Shelesnyak demonstrated that estradiol, estrone and estriol cause the disappearance of mast cells from the uterine endometrium thus suggesting a reduction in uterine histamine. Shelesnyak also suggested that a histamine-releasing-fac tor was involved, liberated by estrogens in the decidua cell reaction. In 1962, Shelesnyak, therefore, suggested that the normal stimulus for implantation is a surge of estrogens which release histamine from the mast cells.

Shelesnyak, et. al. have shown that an antiestrogen (MER-25), administered septematically, inhibits nidation presumably by antagonizing the estrogen surge. Also, early injection of an estrogen or antiestrogen could possibly antagonize the progestational state of the uterus making it insensitive to the surge of estrogen and thus, a decrease in implantation sites. Yochim and DeFeo have suggested that nidation is dependent on fairly narrow limits of estrogen and progesterone concentration and any change in the circulating hormone level would result in the interruption of pregnancy. Martin has shown that the histamine-estrogen relationship is indicative to the uterus, for histamine release is not involved in other estrogen sensitive tissues, such as those found in the vagina.
4. The effects of estrogens and antiestrogens on pregnancy after implantation has occurred.

Pregnancy can be interrupted after the completion of implantation by the administration of either estrogens or antiestrogens. It has been noted, however, ever since the early works of Smith in 1926, Kelly in 1931 and D'Amour, et al. in 1933, that as gestation proceeds, greater quantities of estrogens and antiestrogens are required to interrupt pregnancy. Levin, et al. found that estrogens administered in relatively small doses, over a period of 3 days, during the second half of pregnancy, in rats, did not adversely affect either the pregnancy or length of gestation.

In 1938, Parker, et al. found that rabbits given 0.5 mg. or more of ethinylestradiol, an orally active estrogen, 9 to 10 days after mating resulted in resorption of all embryos. Also, rabbits receiving 1.0 mg. of DES on the 13, 14 and 15 days were found to contain 10 embryos, all being reabsorbed. Conversely, a similar dose on the 11, 12, and 13 day and half this dose on the 10, 11 and 12 day in other rabbits did not disturb pregnancy. In 1939, Heckel and Allen found that the administration of estrogen early after implantation resulted in resorption of the rabbit fetuses, but if administered between the twentieth to twenty-seventh day, of the 32 day gestation period, spontaneous abortion occurred. Huggett and Pritchard in 1945, found that after the twelfth day of pregnancy, with involution of decidua and the establishment of the allantoic circulation, low doses of estrone and
DMS can no longer cause fetal death in rats. Jost\(^74\) in 1945, caused resorption and abortion in mice by the injection of 5.0 mg. of testosterone propionate on the eighth day of pregnancy, and Courrier and Jost\(^35\) found that the death of rabbit fetuses occurred when 20 mg. of testosterone propionate was given daily to the mother from 16 to 21 days of gestation. Velardo, et al.,\(^137\) in 1956, found that estriol and certain of the C\(_{21}\)-metabolites related to progesterone (e.g. pregnane-3\(\alpha\)-20\(\beta\)-diol and pregnanedione), administered in later pregnancy to rats, resulted in early abortions, some resorption of fetuses and reduced litter sizes. Velardo and his colleagues suggest that early abortion can be ascribed primarily either to inadequacy of the implantation site or to abnormalities of the embryo, or perhaps a combination of the two\(^137\). In 1960, Fowler and Edwards\(^56\) demonstrated that mice, when injected with progesterone or estradiol benzoate in late pregnancy, resulted in high fetal mortality.

As well as the animal estrogens and antiestrogens, plant estrogens and antiestrogens can result in reproductive disorders in late pregnancy. MacDonald\(^84\) in 1952, demonstrated that the ingestion of pine needles or pine buds, was the causative agent of abortion and the birth of weak beef calves. In 1964, Cook and Kitts\(^33\) attributed the deleterious effects of the pine needle to its antiestrogenic content. Allen and Kitts\(^6\) in 1961, extracted
the estrogenic components from pine needles which were then incorporated into a ration and fed to mice after theoretical implantation had occurred. When autopsied, many of the embryos had been completely resorbed.

5. **Direct Effects of Estrogens and Antiestrogens on Ova and Embryos.**

It has been shown that the natural estrogens\(^\text{104}\) and progesterone\(^\text{140}\) in excessive amounts are neither directly toxic to the ova nor are they essential for normal cleavage up to the blastula stage. Burdick, et. al.,\(^\text{25}\) found that after estrogen injection, the normal round blastocyst stage, in which the trophoblast cells are in contact with the zona pellucida, is usually attained by the developing tubal ova. In 1942, Alden\(^\text{3,4}\) concluded that no special uterine environment is necessary for production of the blastocyst, nor are ovarian secretions necessary for the early stages of the blastocyst formation. In fact, mouse eggs have shown development when placed into the anterior chamber of the eye.\(^\text{110}\)

Antimetabolites, however, such as 6 diazo 5 oxo L-norleucine,\(^\text{111}\) and synthetic estrogens and antiestrogens, such as DES\(^\text{9}\) and MER-25\(^\text{115}\) are cytotoxic to the developing ova. Even after implantation, compounds such as norethynodrel\(^\text{39}\) and others can induce extensive fetal destruction. It is felt that cytotoxic effects of the estrogens and antiestrogens, however, is not due to their respective hormonal activities per se.\(^\text{76}\)
III Materials and Methods

PART A: Feeding Trial

A study extending from April 3, 1967 to May 19, 1967 was designed to observe the effects of daily feeding of DES and a forage antiestrogen extract on female mink during pregnancy. The two hormones were not mixed into the daily ranch ration until implantation of the fertilized egg was assumed to have occurred in the female. Commercial powdered DES was used, while the antiestrogen was extracted from dehydrated ground alfalfa leaf-meal. DES was incorporated into the mink ration, so that the animals received 5, 10, or 15 mcg. per day. The antiestrogen extract was incorporated into the diet so that the mink consumed 5, 10, or 15, grams equivalent of alfalfa per day.

1. Materials
   a. Into a completely randomized design, thirty standard female mink were distributed into 6 groups with 5 animals per group.
   b. Housing - All animals were housed at the U.B.C. mink unit in individual wire cages (17" x 15" x 15"). A wooden next box (8.5" x 7" x 7.5") was attached to one end of the cage and straw was used as bedding.

2. Methods
   a. Feeding - The 6 experimental rations were mixed once every second day. Sufficient amounts of each ration were mixed and weighed into equal portions to permit daily feeding of the rations. The second day's feed was kept refrigerated until fed. To reduce the possibility of DES or antiestrogen contamination in the feed for the remainder of the U.B.C. herd
(the control groups), all mixing was done at a separate location distant to where the control feed was mixed. A wooden cover was placed over the feed on top of the experimental cages to prevent further contamination by physical transfer of feed to the surrounding cages. Water was fed ad libitum.

b. Extraction of antiestrogen - The extraction of the alfalfa antiestrogen was a modification of the method developed by Luizzo, et al.83 (See Appendix I). All alfalfa extractions were stored under refrigeration until mixed into the ranch ration.

c. Antiestrogen activity - The hormonal activity of the antiestrogen was determined using the Astwood, six-hour bioassay8. (See Appendix II)

d. Oral activity of DES - DES was fed to immature female rats for one week. At the end of this period the animals were sacrificed and the uteri were dissected free, trimmed of fat and weighed. The uterine response is expressed as a percentage of body weight.

e. Statistical Analysis:- Treatment effects were analysed using the Analysis of Variance132.

PART B: Feeding Trial

On March 4, 1968 a second experiment was initiated to study the effects of DES fed at various stages of pregnancy. The experiment extended until May 11, 1968. Fifteen mcgms. of DES was fed to 6 groups of female mink according to the following scheme:
Group I For one week pretupping.
Group II From breeding to implantation.
Group III From implantation to the end of gestation.
Group IV From one week pretupping to implantation.
Group V From breeding to the end of gestation.
and Group VI From one week pretupping to the end of gestation.

The alfalfa antiestrogen extract was fed to 3 groups of mink after implantation had occurred at levels as described in Part A of this study.

1. Materials
a. Animals - In the DES study, 70 pastel female mink were distributed randomly into 7 groups with 10 animals per group. The controls received no DES. In the antiestrogen study, 20 pastel mink were randomized into 4 groups with 5 animals per group. One group, the controls, received no antiestrogen.

   b. Housing - All animals were housed as described in Part A of this study.

2. Methods
a. Feeding - All mixing and feeding of the experimental rations were performed according to the procedure described in Part A of this study.

   b. Extraction of antiestrogen - See part A of this study.

   c. Antiestrogen and DES activity - See Part A of this study.

   d. Statistical Analysis - See Part A of this study.
IV Results and Discussion

The estrogenic activity of the DES and the antiestrogenic activity of the alfalfa extract used in Part A and B of Study I were tested before each experiment was initiated. The results of the alfalfa extract bioassays are given (Tables I and II). The uterine response to the hormonal injections is expressed as a percentage of body weight to adjust for the variations in body size. It was found, by means of an Analysis of Variance, that the differences between the average uterine weights in the three treatments of the first bioassay (Table I) were significant ($P < 0.025$). The results of the second bioassay (Part B) were also significant, but to a higher degree ($P < 0.005$). In the bioassay for Part A the average uterine weight as a percent body weight in group III (estradiol-17β + C.E.C.) was lower than the control group. This suggests that the alfalfa antiestrogen not only acted antagonistically to the estradiol 17-β, but caused a dehydration of the uterus, which may be of some significance in its action as an antifertility agent. From the results, therefore, in Tables I and II, it can be said that the alfalfa extract was significantly antiestrogenic.

The oral activity of the DES was also tested. The same source of DES was used for Parts A and B of this study, therefore only one bioassay was done. The results presented in Table III show that the oral administration of DES to immature, ovariectomized Wistar rats caused a significant ($P < 0.05$) increase in uterine weight. Over the range studied, each increase in the level of DES fed resulted in a significant ($P < 0.05$) increase in uterine weight. A plot of the data (Fig. I) shows a typical linear dose/response relationship between the
### TABLE I

**Antiestrogenic Activity of the Alfalfa Extract Used in Study I Part A.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight (gms)</th>
<th>Uterine Weight (mgm)</th>
<th>Uterine Weight (% body wt.)</th>
<th>Average Uterine Weight ± S.E.(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>0.2 ml. physiological saline (0.9% saline) per rat.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>79.1</td>
<td>21.1</td>
<td>0.0267</td>
<td></td>
</tr>
<tr>
<td></td>
<td>82.4</td>
<td>23.8</td>
<td>0.0289</td>
<td></td>
</tr>
<tr>
<td></td>
<td>70.4</td>
<td>22.0</td>
<td>0.0313</td>
<td>0.0325 ± 0.0026</td>
</tr>
<tr>
<td></td>
<td>78.5</td>
<td>27.7</td>
<td>0.0353</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80.3</td>
<td>32.4</td>
<td>0.0403</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>0.025 µg Estradiol 17-β in 0.2 ml 0.9% saline per rat.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>77.4</td>
<td>32.8</td>
<td>0.0424</td>
<td></td>
</tr>
<tr>
<td></td>
<td>85.6</td>
<td>28.1</td>
<td>0.0328</td>
<td></td>
</tr>
<tr>
<td></td>
<td>82.7</td>
<td>36.0</td>
<td>0.0435</td>
<td>0.0382 ± 0.0026</td>
</tr>
<tr>
<td></td>
<td>85.8</td>
<td>26.5</td>
<td>0.0309</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72.5</td>
<td>30.1</td>
<td>0.0415</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>0.025 µg Estradiol 17-β + CEC (10 gm D.M./rat) in 0.2 ml 0.9% saline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>77.4</td>
<td>20.4</td>
<td>0.0264</td>
<td></td>
</tr>
<tr>
<td></td>
<td>68.1</td>
<td>16.9</td>
<td>0.0248</td>
<td></td>
</tr>
<tr>
<td></td>
<td>83.6</td>
<td>23.5</td>
<td>0.0281</td>
<td>0.0271 ± 0.0019</td>
</tr>
<tr>
<td></td>
<td>90.3</td>
<td>22.7</td>
<td>0.0251</td>
<td></td>
</tr>
<tr>
<td></td>
<td>83.2</td>
<td>26.0</td>
<td>0.0313</td>
<td></td>
</tr>
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</table>

\(^a\)S.E. = Standard Error
# TABLE II

**Antiestrogenic Activity of the Alfalfa Extract used in Study I Part B.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight (gm)</th>
<th>Uterine Weight (mgm)</th>
<th>Uterine Weight (% Body Wt.)</th>
<th>Average Uterine Wt. ± S.E. a</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Controls 0.2 ml physiological saline (0.9% saline)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>59.8</td>
<td>22.0</td>
<td>0.0368</td>
<td></td>
</tr>
<tr>
<td></td>
<td>60.6</td>
<td>16.0</td>
<td>0.0264</td>
<td></td>
</tr>
<tr>
<td></td>
<td>67.8</td>
<td>25.0</td>
<td>0.0369</td>
<td>0.0320 ± 0.0026</td>
</tr>
<tr>
<td></td>
<td>75.0</td>
<td>23.0</td>
<td>0.0307</td>
<td></td>
</tr>
<tr>
<td></td>
<td>57.2</td>
<td>22.0</td>
<td>0.0385</td>
<td></td>
</tr>
<tr>
<td></td>
<td>61.5</td>
<td>14.0</td>
<td>0.0228</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>0.025 μg Estradiol 17-β in 0.2 ml 0.9% saline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>63.7</td>
<td>32.0</td>
<td>0.0502</td>
<td></td>
</tr>
<tr>
<td></td>
<td>68.3</td>
<td>34.0</td>
<td>0.0498</td>
<td></td>
</tr>
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<td></td>
<td>69.7</td>
<td>25.0</td>
<td>0.0359</td>
<td>0.0451 ± 0.0021</td>
</tr>
<tr>
<td></td>
<td>53.7</td>
<td>24.0</td>
<td>0.0447</td>
<td></td>
</tr>
<tr>
<td></td>
<td>73.0</td>
<td>34.0</td>
<td>0.0466</td>
<td></td>
</tr>
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<td></td>
<td>74.5</td>
<td>32.5</td>
<td>0.0436</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>0.025 μg Estradiol 17-β + C.E.C. (10 gm D.M./rat) in 0.2 ml 0.9% saline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>59.9</td>
<td>22.0</td>
<td>0.0367</td>
<td></td>
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<tr>
<td></td>
<td>63.0</td>
<td>19.0</td>
<td>0.0302</td>
<td></td>
</tr>
<tr>
<td></td>
<td>52.2</td>
<td>21.0</td>
<td>0.0402</td>
<td>0.0337 ± 0.0016</td>
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<tr>
<td></td>
<td>51.1</td>
<td>16.5</td>
<td>0.0323</td>
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<td>50.2</td>
<td>15.0</td>
<td>0.0299</td>
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<td></td>
<td>47.1</td>
<td>15.5</td>
<td>0.0329</td>
<td></td>
</tr>
</tbody>
</table>

a S.E. = Standard Error
TABLE III

The Estrogenic Activity of DES Administered Orally to Weanling Female Wistar Rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight (gm)</th>
<th>Uterine Weight (mgm)</th>
<th>Uterine Weight (% Body Wt.)</th>
<th>Average Uterine Wt. ± S.E. a</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Controls: 0 mcgm DES/rat/week.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>57.3</td>
<td>25.6</td>
<td>0.0447</td>
<td></td>
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<td></td>
<td>68.2</td>
<td>33.8</td>
<td>0.0496</td>
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</tr>
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<td></td>
<td>71.4</td>
<td>18.9</td>
<td>0.0265</td>
<td></td>
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<td></td>
<td>72.0</td>
<td>23.0</td>
<td>0.0319</td>
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<td>53.3</td>
<td>20.8</td>
<td>0.0390</td>
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<td></td>
<td>68.1</td>
<td>19.1</td>
<td>0.0280</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>0.150 mcgm DES/rat/week</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>72.1</td>
<td>30.2</td>
<td>0.0419</td>
<td></td>
</tr>
<tr>
<td></td>
<td>66.6</td>
<td>22.1</td>
<td>0.0332</td>
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<td></td>
<td>52.4</td>
<td>28.5</td>
<td>0.0544</td>
<td></td>
</tr>
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<td>63.0</td>
<td>18.4</td>
<td>0.0292</td>
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<td></td>
<td>67.0</td>
<td>40.8</td>
<td>0.0609</td>
<td></td>
</tr>
<tr>
<td></td>
<td>58.4</td>
<td>23.8</td>
<td>0.0408</td>
<td></td>
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<tr>
<td>III</td>
<td>0.300 mcgm DES/rat/week</td>
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<td>67.3</td>
<td>43.0</td>
<td>0.0639</td>
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<td>60.1</td>
<td>38.1</td>
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<td>64.6</td>
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<td>56.8</td>
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<td>70.2</td>
<td>33.1</td>
<td>0.0472</td>
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<td>IV</td>
<td>0.350 DES/rat/week</td>
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<td>45.8</td>
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<td>71.5</td>
<td>40.0</td>
<td>0.0559</td>
<td></td>
</tr>
<tr>
<td></td>
<td>62.4</td>
<td>46.2</td>
<td>0.0740</td>
<td></td>
</tr>
<tr>
<td></td>
<td>59.0</td>
<td>21.5</td>
<td>0.0364</td>
<td></td>
</tr>
</tbody>
</table>

aS.E. = Standard Error
Fig. 1. To show the dose/response relationship between oral administration of DES and uterine weight of an immature, ovariectomized Wistar Rat.
level of DES fed and the uterine response. The response can only be said to be linear within the range studied, because further increases in the level of DES fed would eventually show a curvilinear relationship as a maximal uterine response was approached.

In Part A of this study the DES and antiestrogen were not fed to the mink until implantation had occurred. The length of gestation in the mink varies from 42 to 79 days with an average of 51 days. The embryos implant about 28 to 30 days before parturition. The DES and antiestrogen, therefore, were not incorporated into the mink's diet until 30 days after coitus. It was assumed that implantation had occurred in all animals by this time.

A definite decrease in the number of kits per female occurred when DES or the antiestrogen was incorporated into the diet (see Table IV). The levels of 5, 10, and 15 mcg. DES or the plant antiestrogen at levels of 5, 10, and 15 gm. equivalent of alfalfa were not sufficient to produce a complete reproductive failure in any one group. In each group, however, some females were barren at the end of the gestation period. It has been demonstrated many times that as gestation proceeds greater quantities of estrogenic and antiestrogenic compounds are required to interrupt pregnancy and for some animals in this study the levels of hormones used were not sufficient to cause complete reproductive failures. Other factors which would contribute to variations in the severity of hormonal administration, include:

1. individual sensitivity to hormonal actions,
2. variations in the stage of gestation,
3. variations in feed intake which would cause a variation in the level of hormonal intake.
TABLE IV

Reproductive Performance of Mink Receiving DES or Antiestrogen after Ova Implantation - Part A

<table>
<thead>
<tr>
<th>Group</th>
<th>DES Level (mcg/mink/day)</th>
<th>No. of Females Mated</th>
<th>No. of Litters Born</th>
<th>Total Kits Born</th>
<th>No. Kits Per Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>7</td>
<td>1.4</td>
</tr>
<tr>
<td>II</td>
<td>10</td>
<td>5</td>
<td>2</td>
<td>8</td>
<td>1.6</td>
</tr>
<tr>
<td>III</td>
<td>15</td>
<td>5</td>
<td>4</td>
<td>15</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Antiestrogen Level (gm/mink/day)

<table>
<thead>
<tr>
<th>Group</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5</td>
</tr>
<tr>
<td>II</td>
<td>10</td>
</tr>
<tr>
<td>III</td>
<td>15</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
</tbody>
</table>

1 Antiestrogen extract incorporated in equivalent of grams of alfalfa
In 1960, Travis\textsuperscript{136}, in a similar study of DES feeding, found almost complete failure of the normal reproductive processes. As well as a lowered kit production, Travis found reabsorption of litters, lower kit weights and greater kit mortality in mink receiving the DES. Spontaneous abortion was neither reported by Travis\textsuperscript{136} nor observed in the present study.

Part B of this study was initiated the following breeding season (March, 1968). It was thought possible, after studying the data of the previous experiment (Part A), that a dose/response relationship could exist between the level of hormone administered and kit production. No such relationship was evident in the DES groups of Part A, but there is a possibility of such a relationship in the antiestrogen groups. The antiestrogen experiment was, therefore, repeated the following breeding season and to gain further knowledge of the effects of DES on reproduction, 15 mcg. of DES was fed at various stages of pregnancy. The results of Part B are given in Table V.

The feeding of DES during any of the various stages of pregnancy described was catastrophic (Table V). In Group III of the DES experiment, one female died before the completion of the study. Upon necropsy, 6 fetuses were found in utero, with 2 fetuses partially reabsorbed. None of the other animals were sacrificed for necropsy.

Feeding DES one week before breeding (Group I, IV, and VI) prevents ovulation by chemically antagonizing the secretion of the gonadotrophins\textsuperscript{72}. The inhibition of the gonadotrophins prevents oogenesis and subsequent ovulation of the ovum\textsuperscript{112}. When DES is fed immediately after mating (Groups II and V the hormone disrupts the
transport of the ova through the Fallopian tubes. It has been recently suggested by Humphrey, that low doses of an estrogen can cause both tube-locking of the ova at the isthmo-uterine or ampulla-isthmus junction as well as acceleration of the tubal ova. If the fertilized ova reach the uterus normally, the feeding of DES at this time would disrupt the hormonal concert associated with pregnancy and prevent implantation. If DES is not fed until the ova are implanted (Group III), reabsorption or spontaneous abortion of the fetuses may result.

In the antiestrogen study of Part B, it can be seen from the results in Table V, that a reduction in productivity occurred when the antiestrogen was incorporated into the ration. The results, however, suggest that no dose/response relationship exists between the level of antiestrogen fed and the number of kits born per female. In 1962, Emmens and Finn could not find any dose/response relationship between progesterone or ethyl-19-nortestosterone and litter size by either local or parenteral administration in pregnant rats and mice.

From the results of Study I it is evident that DES at levels as low as 5 to 15 mcgm/mink/day can cause complete reproductive failure. At the 15 mcgm level it is possible to prevent or disrupt pregnancy if the hormone is administered for at least one week from one week before breeding to 30 days before parturition. The forage antiestrogen can also reduce the productivity of the mink even when fed after implantation has occurred. It is not as effective, however, as an antifertility agent as is DES. The present experiments show that the alfalfa antiestrogen, concentrated into an extract, is capable of interrupting pregnancy, however its concentration in the plant itself may be so low
### TABLE V

Reproductive Performance of Mink Receiving DES at Various Stages of Pregnancy or Antiestrogen after Implantation

<table>
<thead>
<tr>
<th>Group</th>
<th>DES Level (mcg/mink/day)</th>
<th>No. of Females Mated</th>
<th>No. of Litters Born</th>
<th>Total Kits Born</th>
<th>No. of Kits per Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>15</td>
<td>10</td>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>II</td>
<td>15</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>III</td>
<td>15</td>
<td>10</td>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4</td>
<td>0.4</td>
</tr>
<tr>
<td>IV</td>
<td>15</td>
<td>10</td>
<td>1</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>V</td>
<td>15</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VI</td>
<td>15</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>10</td>
<td>7</td>
<td>49</td>
<td>4.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antiestrogen Level&lt;sup&gt;c&lt;/sup&gt; (gm/mink/day)</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>18</td>
<td>21</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>3.6</td>
<td>4.2</td>
<td>5.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> One kit born dead

<sup>b</sup> One animal in Group III died and upon necropsy, 6 fetuses were found in utero

<sup>c</sup> Antiestrogen extract incorporated in equivalents of grains of alfalfa
that the antiestrogen level in a mink ration from natural sources is of no significance.
V Conclusions

1. The daily feeding of 5, 10 or 15 mcg. of DES for one week, to female mink, at any stage of the reproductive process (from one week before breeding to immediately after implantation) can cause complete reproductive failure.

2. The feeding of a forage (alfalfa) antiestrogen extract at levels equivalent to 5, 10 or 15 gm. of alfalfa to pregnant mink after implantation has occurred will result in a fewer number of kits per litter than mink not receiving the antiestrogen extract.

3. The feeding of various levels of DES or the antiestrogen extract does not adhere to a dose/response relationship either in interrupting pregnancy or reducing the number of kits per female.

4. DES, fed at levels of 5, 10 or 15 mcgm. per mink per day is a more potent anti-fertility agent than the forage antiestrogen fed at levels equivalent to 5, 10 or 15 gm. of alfalfa.
STUDY II

The Effects of Various Protein and Energy Levels on the Maintenance and Early Growth of Mink, (Mustela vison).

PART A. Maintenance

PART B. Early Growth
I. Introduction:

Among the various nutritional interrelationships affecting the dynamic state of the complex organism, the relationship between protein metabolism and energy has been the most extensively investigated. Protein has two functions within the animal body. Firstly, it is essential for the synthesis of body tissues and secondly, it is a source of energy. However, it must be remembered that if the amino acid constituents of the fed protein are used for tissue synthesis, they are not supplying energy for metabolic processes; and if they are supplying energy, they are not available for tissue synthesis.

It is well established that the dietary energy level has a profound effect on food consumption. Sibbald, et al., have shown that 74% of the variation in food consumption in rats is associated with the apparent digestible energy content of the ration. Within physiological limits, the need to satisfy energy requirements evidently provides the primary stimulus to food intake. It is understandable, therefore, that the dietary requirements for nutrients and the efficiency of nutrient utilization are closely related to the intake of energy.

The primary need of the body is for energy, and its priority over the need for protein synthesis should be assumed, since synthesis is an energy requiring process. The primary function of protein, tissue synthesis, can take place only if the energy needs of the organism are provided for.
II. Literature Review

A. Relationship of protein utilization to energy intake.

1) The effects of constant protein levels with varying energy intake.

Protein utilization in an adult individual on an adequate protein intake and nitrogen equilibrium will respond to changes in the energy content of the diet. From studies on undernutrition it is apparent that withdrawal of energy in the form of either carbohydrate or fat results in a decrease in nitrogen retention evident by an impaired nitrogen balance. Animals restricted in energy intake below a maintenance level fall into a negative nitrogen balance and the degree of negativity is directly related to the severity of the caloric restriction. With sufficient fat and labile protein stores, the increase in nitrogen excretion on a caloric deficient diet is a result of the animal catabolizing its labile protein reserves to provide sufficient energy for the essential anabolic functions. During the initial stages of caloric restriction, the nitrogen balance index, which is a function of the nitrogen retained in the body of the animal and is defined as the rate of change of nitrogen balance with respect to absorbed nitrogen, is unchanged. If the degree of caloric depletion is not too marked, the initial rapid loss of body nitrogen tapers off, which indicates that animals are capable of adapting to caloric restrictions by reducing their catabolic activity. In the growing animal, Bosshardt estimated that a 34 percent decrease in energy intake results in a 14 percent decrease in energy expenditure. This shows that
in the growing animal on a constant protein intake energy conservation is accomplished by a reduction in basal metabolism. If the caloric restriction is marked, however, the animal becomes severely depleted in fat and tissue protein reserves and the nitrogen balance index is reduced and the catabolism of tissues is again increased eventually culminating in death of the animal.

The resistance to caloric restriction is correlated, in part, with the magnitude of the protein stores and the caloric reserves of the body. Rosenthal and Allison found that there were differential changes in body tissues in animals on caloric deficient diets. This demonstrated that some organs, such as the liver, were more labile to neutral lipid and protein depletion than other organs, such as the heart. The response, therefore, to a caloric restriction is a function of the physiological state of the animal as well as the nature of the diet.

Energy has a sparing effect on protein metabolism. As caloric intake increases with protein intake constant, protein utilization increases to a maximum, beyond which no additional protein utilization occurs. Calloway and Spector found that for young essentially normal active men, in negative nitrogen balance, when no protein is fed, the protein deficit can be maximally reduced by supplying about 700 non-protein calories. No significant protein-sparing is achieved by intakes as high as 2000 Kcal in the absence of protein. When the diet provides adequate amounts of protein, increments in energy intake, produced by adding carbohydrate or fat to a sub-maintenance diet, causes a linear
improvement in nitrogen balance through equilibrium to a considerable nitrogen retention. Protein metabolism must thus be in a state of dynamic equilibrium with energy intake, even when the animal is in nitrogen equilibrium. Munro and Naismith also found that the total amount of protein contained in the liver responded to increasing energy intake in a linear fashion. Changes in energy intake caused a smaller percentage change in the nitrogen content of the carcass than in the nitrogen content of the liver.

Once a certain energy intake has been reached on a protein-free diet additional increases in energy intake will not lead to further reduction in nitrogen output. This means that when the supply of amino acids circulating to the tissues comes solely from endogenous sources, this becomes a limiting factor in the rate of protein synthesis at quite low levels of energy intake. When the diet supplies adequate protein this limitation is no longer present. However, the major factor limiting growth throughout a wide range of caloric intakes is still the amount of protein ingested. During pregnancy, Pike, et al have shown that in the rat the limiting factor of nitrogen retention during organogenesis was the supply of non-protein calories, rather than the level of protein. However, during the period of rapid fetal growth calories are no longer the factor limiting nitrogen retention; rather, it is the level of nitrogen in the diet that is imposing the limitation. It is possible that there is a caloric intake for each protein intake, the perfect balance between protein and calories resulting in an adequate development of body mass.
2) The effects of constant energy levels with varying protein intake.

Although the efficiency of protein utilization, in conditions of calorie undernutrition, is governed largely by the extent of the caloric restriction; within limits, increasing amounts of protein can be utilized by the body if the protein intake is increased while the non-protein caloric content of the ration is kept constant. As the protein increases with caloric intake constant, protein utilization rises sharply to a peak rate and the rise becomes progressively less. Rosenthal found that dogs fed restricted diets utilized nitrogen in a normal fashion when the protein content was relatively low. Additional dietary protein resulted in a small positive nitrogen balance which could not be increased further no matter how much protein is included in the diet.

The net protein utilization, however, decreases linearly as the percentage of protein calories in the diet is increased, and the rate of decrease is a characteristic of each protein. Calloway and Spector have shown that the nitrogen utilization in rats diminished from approximately 55 to 17 percent when the level of dietary nitrogen increased from 75 to 604 mg. daily. Thus indicating an increased use of dietary protein for energy.
B. The effects of carbohydrate and fat on protein utilization.

Although the caloric intake exerts a significant effect on protein utilization, the difference in response to carbohydrate and fat feeding clearly indicates that carbohydrate also exerts an effect separate from its calorigenic function. On diets that are adequate in protein and energy, replacement of carbohydrate calories by fat calories produces a transient increase in nitrogen excretion which then declines. Furthermore, in complete protein starvation carbohydrate, but not fat, has the property of sparing body protein. Munro and Naismith have shown that in rats on a protein-free diet, the addition of fat to bring the energy intake up from 900 to 1700 Kcal/m$^2$ failed to influence nitrogen balance. The addition of carbohydrate produced some improvement up to 1200 Kcal/m$^2$, but not thereafter. In the case of the rat, the separation of the time of feeding dietary protein and carbohydrate (but not fat) has an adverse effect on nitrogen balance, which is of short duration. These observations may be taken as indicating that carbohydrate does play a part in protein metabolism which cannot be taken by fat.

Munro, et al have suggested that the mechanism through which carbohydrate has an effect of protein utilization may be related to the fact that dietary carbohydrate, but not fat, causes a temporary fall in plasma amino acids and the deposition of amino acids in the muscle. The mechanism causing the shift of amino acids into muscle has been attributed to the action of insulin.
As a result of the reduced plasma amino acid level, the supply of amino acids to other tissues is curtailed and with two consequences:

(a) Urea production by the liver is reduced (protein-sparing action), and,

(b) Tissues other than muscle have a diminished supply of amino acids for protein syntheses.  

Whether the insulin accelerates the transport of amino acids across the muscle cell membrane making the amino acids available to the protein synthetic mechanism within the cell or whether the effect is on the polypeptide synthesis within the cell has not been clarified.
III Materials and Methods

PART A: Feeding Trial

An experiment, extending from November 3, 1966 to December 7, 1966 was designed using a simple randomized block design to study the effects of feeding regular ranch rations varying on the level of protein and energy during the non-critical period of feeding. The protein and energy content of the rations were regulated by altering the protein and energy content of the cereal portion.

1. Materials

   a. Animals - Two hundred mature male and female mink were divided into 5 groups with 20 males and 20 females per group. Due to the restricted availability of any one colour phase of mink, a variety of colour phased animals were used. An attempt, therefore, was made to distribute the different colour phases equally within each group to equalize any colour phase interaction between the groups.

   b. Housing - All animals were individually housed in wire cages (17" x 15" x 15") equipped with a wooden nest box (8.5" x 7" x 7.5") and a water cup. All cages were adequately sheltered at the U.B.C. mink unit.

   c. Feed - Each of the 5 groups received similar rations, (Table VI) which varied only in the cereal mix used. The cereal mixes (Tables VII and VIII) incorporated into the rations differed in level of protein and energy, namely in the form of fat. All rations were mixed and fed daily
TABLE VI

GENERAL COMPOSITION OF RATIONS

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount (Per Cent)</th>
<th>Price (c/lb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>M-1A</td>
</tr>
<tr>
<td>Cereal Mix¹</td>
<td>25.8</td>
<td>6.86</td>
</tr>
<tr>
<td>Fish Scraps</td>
<td>25.8</td>
<td></td>
</tr>
<tr>
<td>Chicken Wastes</td>
<td>25.8</td>
<td></td>
</tr>
<tr>
<td>Horse Meat</td>
<td>17.5</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>Brewer's Yeast</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Vitamin Premix²</td>
<td>80 gms/100lbs of ration - 120.0</td>
<td></td>
</tr>
<tr>
<td>Salt Mix</td>
<td>20 gms/100lbs of ration - 3.1</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>15lbs. of H₂O added per 85lbs. of above mixed ingredients.</td>
<td></td>
</tr>
</tbody>
</table>

Cost of Ration (c/lb) (as fed)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>M-1A</th>
<th>M-1B</th>
<th>M-1C</th>
<th>M-1D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.90</td>
<td>6.08</td>
<td>6.23</td>
<td>5.96</td>
<td>5.81</td>
</tr>
</tbody>
</table>

1. See Tables VII and VIII.

2. Per gram: Vitamin A 550 I.U., Vitamin D 110 I.U., Vitamin E (d, alpha tocopheryl acetate) 0.51 I.U., Citrus Bioflavonoid compound 3.5 mg., Methionine 6.3 mg., Choline 6.3 mg., Inositol 2.1 mg., Thiamine HCl 0.1 mg., Riboflavin 0.3 mg., Pyridoxine 0.03 mg., Vitamin B₁₂ 0.4 mg., Niacin 1.2 mg., d, pantothenic acid 0.1 mg., Tricalcium phosphate 48.0 mg., potassium bi-phosphate 22.0 mg., Sodium chloride 17.7 mg.
### TABLE VII

**THE COMPOSITION OF THE CONTROL CEREAL OF PART A**

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount (lbs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn Meal</td>
<td>350</td>
</tr>
<tr>
<td>Ground Barley</td>
<td>200</td>
</tr>
<tr>
<td>Ground Wheat</td>
<td>335</td>
</tr>
<tr>
<td>Bran</td>
<td>100</td>
</tr>
<tr>
<td>Shorts</td>
<td>100</td>
</tr>
<tr>
<td>Soybean Meal (44%)</td>
<td>330</td>
</tr>
<tr>
<td>Fishmeal (71%)</td>
<td>60</td>
</tr>
<tr>
<td>Meatmeal (50%)</td>
<td>285</td>
</tr>
<tr>
<td>Vita Grass</td>
<td>40</td>
</tr>
<tr>
<td>Tomatoe Pomace</td>
<td>50</td>
</tr>
<tr>
<td>Brewer's Yeast</td>
<td>40</td>
</tr>
<tr>
<td>Distillers' Dried Solubles</td>
<td>40</td>
</tr>
<tr>
<td>Stabilized Fat</td>
<td>55</td>
</tr>
<tr>
<td>Salt</td>
<td>10</td>
</tr>
<tr>
<td>Vitamin Premix</td>
<td>5</td>
</tr>
</tbody>
</table>
TABLE VIII
THE COMPOSITION OF EXPERIMENTAL CEREAL MIXES OF PART A

<table>
<thead>
<tr>
<th>Constituents</th>
<th>M-1A (lbs)</th>
<th>M-1B (lbs)</th>
<th>M-1C (lbs)</th>
<th>M-1D (lbs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground Wheat</td>
<td>300</td>
<td>300</td>
<td>525</td>
<td>650</td>
</tr>
<tr>
<td>Ground Oats</td>
<td>300</td>
<td>300</td>
<td>525</td>
<td>650</td>
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<td>Brewers' Yeast</td>
<td>60</td>
<td>60</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Salt</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Herring Meal</td>
<td>600</td>
<td>600</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Soybean Oil Meal</td>
<td>500</td>
<td>500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood Meal</td>
<td>80</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meat Meal</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Bone Meal</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Stabalized Fat</td>
<td></td>
<td>180</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>Vitamin Premix(^1)</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

1. Dry Vitamin A 1,000,000 I.U., Vitamin D 240,000 I.U., Vitamin E 28 gms., Thiamine mononitrate 2 gms., Riboflavin 7 gms., Niacin 12 gms., Pyridoxine 5 gms., Biotin 0.1 gms., Folic Acid 1.5 gms., Manfanese Sulfate 18.0 gms., Dacal 6.0 lbs., Alfalfa leafmeal 93.0 lbs.
in the afternoon. The raw meats were stored at a temperature of approximately -20°C. Twenty-four hours prior to mixing, the daily allotments of meat were removed from the freezer and allowed to thaw. Water was supplied *ad libitum*.

2. **Methods**

   a. **Animals** — The day prior to the feeding of the experimental rations, all mink were individually weighed and their body weights recorded. At the completion of the experiment all mink were sacrificed by an interperitoneal injection of 1 ml. of nicotine sulfate (20% solution) and weighed.

   b. **Feeds** — The percentage dry matter, protein and ether extract of the cereals and total rations were determined using the official A.O.A.C. methods.

   c. **Statistical Analysis** — Initial body weight, final body weight, and body weight gain were analysed using the Analysis of Variance and Duncan's New Multiple Range Test.

**PART B**

1. **Feeding Trial**

   To gain additional information on the protein/Calorie requirements of mink, an experiment, extending from July 8, 1967 to October 31, 1967 was designed (simple randomized block design) to study the effects of feeding rations of varying protein and energy levels to mink kits.
during the critical period of feeding. The protein and energy content of the total rations were again regulated by using cereal mixes with different protein and energy contents.

a. Materials

i. Animals - Two hundred and eighty pastel and standard kits were randomly distributed into 7 groups with 40 kits per group. Twenty males and twenty females were in each group.

ii. Housing - All animals were housed in cages as described in Part A of this study. Due to the lack of space, however, one-half of the animals on experiment had to be housed two per cage. The nest boxes were removed from the cages on approximately August 1, 1967.

iii. Feed - The cereal mixes were incorporated into rations identical in composition to the rations given in Part A (Table VI) of this study. The cereal mixes (Table IX) varied in protein and energy content as in the previous experiment, however, in this study the protein and calorie density of the rations were increased to allow for the increase nutritive demands of growth. The preparation and mixing of the experimental rations is described in Part A of this study.

b. Methods

Data collection, feed and statistical analysis are described in Part A of this study. One method included was the gross energy determination of the total rations and cereal mixes using a Parr oxygen bomb calorimeter. Theremethod is described in the Parr technical manual No. 130100.
TABLE IX
THE COMPOSITION OF EXPERIMENTAL CEREAL MIXES OF PART B

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground Wheat</td>
<td>420</td>
<td>370</td>
<td>250</td>
<td>445</td>
<td>430</td>
<td>170</td>
<td>450</td>
</tr>
<tr>
<td>Ground Oats</td>
<td>525</td>
<td>370</td>
<td>250</td>
<td>550</td>
<td>500</td>
<td>500</td>
<td>450</td>
</tr>
<tr>
<td>Brewers' Yeast</td>
<td>50</td>
<td>35</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Salt</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Herring Meal (71%)</td>
<td>300</td>
<td>725</td>
<td>800</td>
<td>500</td>
<td>540</td>
<td>465</td>
<td>550</td>
</tr>
<tr>
<td>Soybean Oil Meal (48.5%)</td>
<td>425</td>
<td>250</td>
<td>350</td>
<td>250</td>
<td>250</td>
<td>200</td>
<td>250</td>
</tr>
<tr>
<td>Meatmeal</td>
<td>90</td>
<td>90</td>
<td>140</td>
<td>140</td>
<td>100</td>
<td>360</td>
<td>90</td>
</tr>
<tr>
<td>Bonemeal</td>
<td>55</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>40</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>Stabalized Fat</td>
<td>115</td>
<td>100</td>
<td>100</td>
<td>5</td>
<td>70</td>
<td>135</td>
<td>100</td>
</tr>
<tr>
<td>Vitamin Premix&lt;sup&gt;1&lt;/sup&gt;</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

1. Per Ton: Vitamin A 4,800,000 I.U., Vitamin D 240,00 I.U., Vitamin E 28.0 gm., Thiamine 3.2 gm., Riboflavine 5.4 gm., Pantothenie acid 22.4 gm., Nicotine Acid 27.5 gm., Pyridoxine 3.2 gm., Folic acid 0.5 gm.
2. **Digestibility Trials**

Two digestibility trials, each of a 7 day duration, were carried out on the rations used in Part B of Study II. The total collection method was used with the adaptation period lasting 3 days and the collection period continued for 4 days. Each day of the collection period the feces and urine excreted and feed consumed were recorded. The feces was collected on a wire screen and the urine in a 125 ml. flask containing 2 ml. of 50% H_2SO_4. The urine and feces were pooled for analysis.

a. **Materials**

i. **Animals** - In each trial, 7 male and 7 female pastel (Trial 1) or Standard (Trial 2) adult mink were transferred to a digestibility cage. This was designated as Day 1 of the digestibility trial. The animals were weighed before and after the 7 day adaption and collection period.

ii. **Digestibility Cages** - The cages used were constructed to the specifications as outlined by Sinclair and Evans. Before each digestibility trial the cages were thoroughly cleaned with detergent and hot water and scrubbed with a wire brush.

iii. **Feeds** - The frozen meats used in the rations were allowed to thaw for twenty-four hours prior to grinding. To ensure uniformity of the feed and also to prevent the mink from carrying large pieces of feed away from the feeding area, the meats were put through a grinder twice. Sufficient quantities of the rations were mixed for the entire trial on Day 1 and stored under refrigeration until fed. Each ration was fed to one male and one female mink.
b. Methods

All nitrogen, ether extract and dry matter determinations were done using the official A.O.A.C. procedures\textsuperscript{67}. Gross energy of the feeds and feces were determined by oxygen bomb calorimetry\textsuperscript{100}.
IV. Results and Discussion

The mean initial, final, and body weight gains of the animals used in Part A of Study II are shown in Table X. A complete record of initial and final body weights is given in Appendix III. For the purpose of this experiment the criterion for a maintenance ration was defined as that ration which, when fed ad libitum, caused neither a significant increase nor decrease in initial body weight. The levels of protein and fat of the various rations are given in Table XI. For purposes of clarity, the four experimental rations are classified as follows: a) low protein/low fat, M-1D; b) low protein/high fat, M-1C; c) high protein/low fat, M-1A; and d) high protein/high fat, M-1B. The ration classified as the control was the U.B.C. ranch ration (1960 to 1966) and was employed in this experiment for the purpose of comparison.

<table>
<thead>
<tr>
<th>Ration</th>
<th>Initial Body Wt. (gm.)</th>
<th>Final Body Wt. (gm.)</th>
<th>Body Wt. Gain (gm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1203</td>
<td>1218</td>
<td>15</td>
</tr>
<tr>
<td>M-1A</td>
<td>1233</td>
<td>1311</td>
<td>79</td>
</tr>
<tr>
<td>M-1B</td>
<td>1263</td>
<td>1339</td>
<td>76</td>
</tr>
<tr>
<td>M-1C</td>
<td>1260</td>
<td>1338</td>
<td>79</td>
</tr>
<tr>
<td>M-1D</td>
<td>1220</td>
<td>1218</td>
<td>-2</td>
</tr>
</tbody>
</table>
TABLE XI. Proximate Analysis of Rations\textsuperscript{a} for Study II, Part A.

<table>
<thead>
<tr>
<th>Cereal Mix</th>
<th>Percent Dry Matter</th>
<th>Percent Protein (D.M. Basis)</th>
<th>Percent Ether Extract</th>
<th>Total Ration</th>
<th>Percent Dry Matter</th>
<th>Percent Protein (D.M. Basis)</th>
<th>Percent Ether Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>88.56</td>
<td>27.02</td>
<td>10.19</td>
<td>30.75</td>
<td>43.03</td>
<td>23.76</td>
<td></td>
</tr>
<tr>
<td>M-1A</td>
<td>84.13</td>
<td>40.96</td>
<td>7.42</td>
<td>34.62</td>
<td>44.15</td>
<td>24.20</td>
<td></td>
</tr>
<tr>
<td>M-1B</td>
<td>88.46</td>
<td>37.27</td>
<td>19.87</td>
<td>35.27</td>
<td>44.75</td>
<td>28.87</td>
<td></td>
</tr>
<tr>
<td>M-1C</td>
<td>84.02</td>
<td>26.05</td>
<td>19.05</td>
<td>33.47</td>
<td>40.57</td>
<td>27.17</td>
<td></td>
</tr>
<tr>
<td>M-1D</td>
<td>83.68</td>
<td>26.71</td>
<td>11.08</td>
<td>32.00</td>
<td>36.64</td>
<td>21.25</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} All values are averages of triplicate determinations.

Greater (P < 0.05) final body weights over the control and M-1D rations, of which ration M-1D is low in protein and fat. Ration M-1A (high protein/low fat) did not produce final body weights which differed significantly (P < 0.05) from either rations M-1D and control or M-1B and M-1C.

The M-1D ration appears to be the most satisfactory ration to maintain mink near a constant body weight. This would suggest that rations containing approximately 36 percent protein and 21 percent fat, on a dry matter basis, are satisfactory for the late growth requirements of mink or over the maintenance period. Rations higher in protein or fat content would subsequently be higher energy rations and as a result, because the animals are in the latter stages of growth, would deposit
excessive amounts of body fat, a condition undesirable for either proper pelt processing or breeding purposes.

In Part B of this study a more detailed investigation was conducted to determine the protein and energy requirements of mink during their growing period. The proximate analyses of the 7 rations are shown in Table XII. The percent protein and fat (ether extract) on a dry matter basis ranged from 38.9 to 54.3 percent and 18.3 to 22.8 percent, respectively. The mean initial body weight, final body weight and body weight gains are given in Table XIII and calculated from the values in Appendix IV. From an analysis of variance it was found that the initial body weights of the mink used in this study

Table XII. Proximate Analysis of Rations  \( ^{a} \) for Study II, Part B

<table>
<thead>
<tr>
<th>Ration</th>
<th>Cereal Mix</th>
<th>Total Ration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent Dry Matter</td>
<td>Percent Protein (D.M. Basis)</td>
</tr>
<tr>
<td>Control</td>
<td>92.18</td>
<td>36.70</td>
</tr>
<tr>
<td>M-2A</td>
<td>92.16</td>
<td>32.11</td>
</tr>
<tr>
<td>M-2B</td>
<td>91.64</td>
<td>42.09</td>
</tr>
<tr>
<td>M-2C</td>
<td>90.85</td>
<td>47.46</td>
</tr>
<tr>
<td>M-2D</td>
<td>92.31</td>
<td>40.49</td>
</tr>
<tr>
<td>M-2E</td>
<td>91.65</td>
<td>37.51</td>
</tr>
<tr>
<td>M-2F</td>
<td>90.23</td>
<td>39.18</td>
</tr>
</tbody>
</table>

\( ^{a} \) Each value is the mean value from triplicate determinations.
Table XIII. Mean Initial, Final, and Body Weight Gains of Study II, Part B

<table>
<thead>
<tr>
<th>Ration</th>
<th>Initial Body Wt. a (gm.)</th>
<th>Final Body Wt. (gm.)</th>
<th>Body Wt. Gain (gm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>553.6</td>
<td>1160</td>
<td>606.7</td>
</tr>
<tr>
<td>M-2A</td>
<td>569.0</td>
<td>1052</td>
<td>482.7</td>
</tr>
<tr>
<td>M-2B</td>
<td>582.9</td>
<td>1124</td>
<td>541.9</td>
</tr>
<tr>
<td>M-2C</td>
<td>583.4</td>
<td>1098</td>
<td>514.2</td>
</tr>
<tr>
<td>M-2D</td>
<td>667.3</td>
<td>1098</td>
<td>430.8</td>
</tr>
<tr>
<td>M-2E</td>
<td>627.7</td>
<td>1016</td>
<td>389.1</td>
</tr>
<tr>
<td>M-2F</td>
<td>660.3</td>
<td>1119</td>
<td>459.3</td>
</tr>
</tbody>
</table>

a Significantly different (P < 0.01)

were significantly different (P < 0.01). The final and initial body weights were therefore tested using the Analysis of Covariance (62). A significant difference (P < 0.01) in final body weight was obtained by this analysis. The ration which caused the greatest gains in weight was the control ration resulting in significantly greater final body weights. The final body weights produced by rations M-2B, M-2C, M-2D and M-2F were not significant (P < 0.05). Ration M-2A did not produce a final body weight significantly different (P < 0.05) from that produced by ration M-2E, but this final body weight was significantly different (P < 0.05) from the final body weights produced by the other rations.
The detailed analysis of the digestibility studies and nitrogen retention calculations are given in Appendices V and VI. Summaries of the digestibility trials are given in Tables XIV and XV.

It would be hazardous to suggest from the limited replications in the digestibility trials that any one ration was superior due to a larger apparent digestibility coefficient. The data in Tables XIV and XV do show, however, definite ranges in apparent digestible dry matter, nitrogen, ether extract and energy coefficients. In both trials the apparent dry matter digestibility of the 7 rations averaged between 61.2 and 71.6%; the percent apparent digestible nitrogen (A.D.N.) averaged between 70.1 and 78.1, the apparent ether extract digestibility averaged between 83.0 and 91.4% and the apparent digestible energy (A.D.E.) varied between 69.6 and 77.9% of the gross energy. It has been repeatedly shown that mink utilize fat efficiently and that fats are digested more efficiently than protein (81). The apparent digestibility coefficients for fat and protein have been reported slightly higher in early growth mink rations than those obtained in this study. For example, Roberts and Kirk (106) obtained apparent digestibility coefficients for fat and protein of 97.96 and 74.82%, respectively. Bernard, et al. (12) obtained values for fat and crude protein (chief source raw horse meat) of 93.0 and 87.0%, respectively. Leoschke (81) corrected the apparent digestibility of fat for the metabolic fat present in the faeces of an early growth ration and obtained a true digestibility coefficient for fat (beef tallow) of 91.9%. The average apparent digestible dry matter in this
Table XIV. Summary of Digestibility Trial I

<table>
<thead>
<tr>
<th>Ration</th>
<th>Percent D.M. Digestibility</th>
<th>Percent Nitrogen Digestibility</th>
<th>Percent Ether Extract Digestibility</th>
<th>Percent Digestible Energy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Control (mean)</td>
<td>66.08</td>
<td>68.20</td>
<td>72.00</td>
<td>82.67</td>
</tr>
<tr>
<td>(mean)b</td>
<td>69.76</td>
<td>78.1</td>
<td>86.81</td>
<td>76.96</td>
</tr>
<tr>
<td>M-2A (mean)</td>
<td>56.14</td>
<td>72.45</td>
<td>69.12</td>
<td>78.42</td>
</tr>
<tr>
<td>(mean)</td>
<td>61.26</td>
<td>(70.1)</td>
<td>86.51</td>
<td>(69.6)</td>
</tr>
<tr>
<td>M-2B (mean)</td>
<td>68.24</td>
<td>73.11</td>
<td>73.93</td>
<td>79.68</td>
</tr>
<tr>
<td>(mean)</td>
<td>71.6</td>
<td>(76.91)</td>
<td>90.10</td>
<td>(77.91)</td>
</tr>
<tr>
<td>M-2C (mean)</td>
<td>66.21</td>
<td>67.44</td>
<td>79.55</td>
<td>80.41</td>
</tr>
<tr>
<td>(mean)</td>
<td>68.7</td>
<td>(76.81)</td>
<td>(87.2)</td>
<td>(76.6)</td>
</tr>
<tr>
<td>M-2D (mean)</td>
<td>68.10</td>
<td>66.82</td>
<td>72.45</td>
<td>84.86</td>
</tr>
<tr>
<td>(mean)</td>
<td>69.81</td>
<td>(77.10)</td>
<td>(83.0)</td>
<td>(77.91)</td>
</tr>
<tr>
<td>M-2E (mean)</td>
<td>63.90</td>
<td>70.78</td>
<td>64.95</td>
<td>81.44</td>
</tr>
<tr>
<td>(mean)</td>
<td>69.31</td>
<td>(72.6)</td>
<td>(91.4)</td>
<td>(76.04)</td>
</tr>
<tr>
<td>M-2F (mean)</td>
<td>69.66</td>
<td>65.08</td>
<td>81.76</td>
<td>76.47</td>
</tr>
<tr>
<td>(mean)</td>
<td>62.71</td>
<td>(71.6)</td>
<td>(85.5)</td>
<td>(75.01)</td>
</tr>
</tbody>
</table>

a. All values are apparent digestibilities
b. Mean of digestibility trial I and II
From the data presented in Tables XIII and XVI, it is evident that as the A.D.E. is raised from 353.4 Kcal/100 gm. dry matter to 426.0 Kcal/100 gm. and as the A.D.N. level reaches 4.980 gm./100 gm. dry matter the early growth of both male and female mink is improved. Maximum performance was obtained in the mink when the ration contained 426.0 Kcal A.D.E./100 gm. dry matter and 4.980 gm. A.D.N./100 gm. dry matter. Sinclair, et. al. (126), in 1962, reported similar results to those obtained in this study. He found that diets high in energy (5.23 Kcal of gross energy/gm; 4.18 Kcal of A.D.E./gm.) were superior to low energy (4.57 Kcal of gross energy/gm; 3.43 Kcal of A.D.E./gm.) diets in promoting early growth and enhancing final body weights in male and female mink.

Table XVI. The Apparent Digestible Energy : Apparent Digestible Nitrogen Ratio for the Experimental Rations as Determined from Digestibility Trial II

<table>
<thead>
<tr>
<th>Ration</th>
<th>Gross Energy (Kcal/100 gm. feed)</th>
<th>Apparent Digestible Energy (Kcal/100 gm. feed)</th>
<th>Total Nitrogen (gm./100 feed)</th>
<th>Cal:Protein Ratio (Kcal/gm.)</th>
<th>Apparent Digestible Nitrogen Ratio (gm.A100 gm. feed)</th>
<th>A.D.E. : A.D.N. Ratio (Kcal/gm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-2A</td>
<td>522.2</td>
<td>353.4</td>
<td>5.520</td>
<td>15.14</td>
<td>3.455</td>
<td>102.29</td>
</tr>
<tr>
<td>M-2B</td>
<td>525.4</td>
<td>407.2</td>
<td>6.914</td>
<td>12.16</td>
<td>5.327</td>
<td>76.44</td>
</tr>
<tr>
<td>M-2C</td>
<td>552.1</td>
<td>420.6</td>
<td>7.061</td>
<td>12.51</td>
<td>5.199</td>
<td>80.90</td>
</tr>
<tr>
<td>M-2D</td>
<td>526.0</td>
<td>406.1</td>
<td>6.221</td>
<td>13.53</td>
<td>4.699</td>
<td>86.42</td>
</tr>
<tr>
<td>M-2E</td>
<td>527.1</td>
<td>414.9</td>
<td>6.955</td>
<td>12.13</td>
<td>5.002</td>
<td>82.95</td>
</tr>
<tr>
<td>M-2F</td>
<td>520.0</td>
<td>362.5</td>
<td>7.004</td>
<td>11.88</td>
<td>4.482</td>
<td>80.88</td>
</tr>
<tr>
<td>Control</td>
<td>535.9</td>
<td>426.0</td>
<td>6.322</td>
<td>13.56</td>
<td>4.980</td>
<td>85.54</td>
</tr>
</tbody>
</table>
Also, diets of high crude protein (4.912 gm. gross N/100 gm; 3.952 gm. A.D.N./100 gm.) were superior to those of low crude protein content (3.760 gm. gross N/100 gm; 2.673 gm. A.D.N./100 gm.) in ability to promote early growth and to enhance final body weights of both sexes.

Sinclair, et. al. (126) also found that the percent digestible nitrogen was curvi-linear related to the A.D.E. : A.D.N. ratio and appeared to be maximal when the diet contained 140 Kcal of A.D.E./gm. A.D.N. The results obtained in this study do not show a curvilinear relationship between percent A.D.N. and the A.D.E. : A.D.N. ratio (Fig. II); however, a maximum response was obtained when the diet contained 85.54 Kcal of A.D.E./gm. A.D.N. (13.6 Kcal gross energy/gm. gross protein).

Although a narrow range of A.D.E. : A.D.N. ratios were studied, results from the nitrogen balance studies (Appendices VI) indicate that the A.D.E. content of the diet had a definite protein-sparing effect (see Table XVII). Maximum nitrogen retention was obtained when the

<table>
<thead>
<tr>
<th>Table XVII. The protein-sparing effect of increasing apparent digestible energy*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ration</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>E</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>D</td>
</tr>
<tr>
<td>F</td>
</tr>
<tr>
<td>A</td>
</tr>
</tbody>
</table>

* All values are averages of 2 replications as determined from digestibility trial II.
Fig. II. Relationship between Percent A. C. N. and the A. D. E. : A. D. N. Ratio.

Apparent Digestible Nitrogen (%)

A. D. E. : A. D. N. Ratio (Kcal/kg)

70.0 80.0 90.0 100.0 110.0
ration contained 426.0 Kcal of A.D.E./100 gm. of dry feed and as the energy level of the ration was reduced more protein was metabolized as a source of energy, indicated by the reduced amount of nitrogen retained.
V. Conclusions

1. Rations containing 35 to 40% crude protein and 20 to 25% fat, on a dry matter basis, are satisfactory for the late growth and maintenance requirements of mink.

2. The mean apparent digestibility coefficients obtained in these studies for dry matter, protein, fat, and energy are 66.38, 74.05, 87.20, and 73.74%, respectively.

3. It is suggested that growing male kits (from 6-28 weeks of age) are capable of utilizing feeds more efficiently than female kits of the same age.

4. As the A.D.E. is raised from 353.4 Kcal/100 gm. dry matter to 426.0 Kcal/100 gm. and as the A.D.N. level reaches 4.980 gm./100 gm. dry matter the early growth of both male and female mink is improved.

5. A maximum early growth rate was obtained in kits when the ration contained 85.54 Kcal of A.D.E./gm. A.D.N. (13.6 Kcal gross energy/gm. gross protein).

6. Increasing the A.D.E. content of the diets had a definite protein-sparing effect. Maximum nitrogen retention was obtained when the ration contained 426.0 Kcal of A.D.E./100 gm. of dry feed.
VI. References cited.


XII APPENDICES
APPENDIX I

ANTIESTROGEN EXTRACTION

Material to be assayed
\[\downarrow\]
Mix with 0.1 N
Autoclave 30 min. at 15 lbs. psi
\[\downarrow\]
Filter and concentrate (heat gently)
Cool, Adjust pH to 7.2
\[\downarrow\]
Precipitate soluble proteins with 2 vols. of 95% ethyl alcohol (let stand for 24 hrs.)
\[\downarrow\]
Filter
\[\downarrow\]
discard ppt.

Aqueous ethyl alcohol mixture
\[\downarrow\]
Concentrate (in vacuo) pH 4.5
\[\downarrow\]
Extract 3x with diethyl ether

Ether-soluble extract (estrogenic)
discard

Aqueous fraction (adjust pH to 4.5)
\[\downarrow\]
Extract 3x with chloroform

Chloroform-soluble extract (Antiestrogenic)
\[\downarrow\]
Aqueous fraction
discard

Concentrate (in vacuo)
Chloroform Extract Concentrate (C.E.C.)
APPENDIX II

Bioassay of Estrogenic and Antiestrogenic Compounds

12

A. Ovariectomy: rat

The animal is placed in a jar and anesthesia is induced with ether. When the animal has ceased to move or struggle, it is removed and placed on an operating board. Hair may or may not be clipped from the site of operation. Usually anesthesia is continued with an ether cone.

The area is cleaned with alcohol and an incision from 1.0 to 1.5 cm. long is made in the skin midway between the last rib and the knee about 1 cm. lateral to the spinal muscles.

A second incision is made through the muscle layer and into the peritoneal cavity. If the incision is made correctly, the ovary will be seen immediately underneath, embedded in a mass of fat. The fat is withdrawn, and the ovary is separated and held with a hemostat. It may then be cut away and the uterus returned to the peritoneal cavity. The incision is then closed by means of a surgical clamp. The ovary on the opposite side may then be removed through a separate incision.

B. Bioassay

Replacement therapy with estrogens restores the reproductive tract of ovariectomized animals to the precastrate state. The degree of growth of the uterus under such therapy is proportional to the dose hormone administered within limits. Thus, the increase
in uterine weight serves as a convenient bioassay for estrogens. Several compounds inhibit the action of estrogens on the reproductive tract and can therefore be tested for their antiestrogenic properties with a similar bioassay as that used for the estrogens.

Immature rats, weighing approximately 60 gm. and which have been ovariectomized two days previously, are distributed into the required number of groups. All injections are made subcutaneously. The control group is injected with physiological saline (0.9% NaCl). The animals are killed with ether 6 hours after injection; the uteri are removed, trimmed of fat, and weighed rapidly on a torsion balance to the nearest 0.1 mg. The uteri are expressed as percent body weight.
## APPENDIX III

### INITIAL & FINAL BODY WEIGHTS OF STUDY II, PART A

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### APPENDIX V

**Digestibility Trial I**

**Apparent Digestibility Dry Matter**

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## Digestibility Trial I

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## Digestibility Trial I

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APPENDIX V (CONTD)

Digestibility Trial I

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Digestibility Trial I

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# APPENDIX VI

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### APPENDIX VI (Contd)

**Digestibility Trial II**

**Apparent Digestible Ether Extract**

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<th>Total Fats Excreted (gms)</th>
<th>Ether Extract (gms)</th>
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Appendix VI (Contd) - Digestibility Trial II

Apparent Digestible Energy

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## APPENDIX VI (Contd)

**Digestibility Trial II - Nitrogen Retention.**

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APPENDIX VII

Analysis of Variance Tables.

Study I

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B. Bioassay 2: Estradiol and C.E.C.

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C. Bioassay of DES.

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### STUDY II

#### PART A.

1. **Initial Body Weight**

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2. **Gain**

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3. **Final Body Weight**

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PART B.

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