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Chemical evidence of the quantitative thiamine and riboflavin requirements of the rat during pregnancy and the development of the fetus

Margaret Louise Barrett
Iowa State College

NOTE TO USERS

CHEMICAL EVIDENCE OF THE QUANTITATIVE THIAMINE AND RIBOFLAVIN REQUIREMENTS OF THE RAT DURING PREGNANCY AND THE DEVELOPMENT OF THE FETUS

by

Margaret Louise Barrett

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Head of Major Department

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INTRODUCTION

Numerous studies in the field of nutrition have suggested a close relationship between the maternal diet and the condition of the child at birth. Faulty diet prior to and during pregnancy is believed to be responsible for several types of disorders encountered at this time. Miscarriages, prematurity, malformations of the young, stillbirths, and toxemias of pregnancy have been noted more frequently among women whose diets were judged inadequate in various essential nutrients than among women with excellent dietary intakes. These statements are borne out by the findings of Ebbs and his associates (1941), Burke, Beal, Kirkwood and Stuart (1943), and Balfour (1944).

It is encouraging that the number of deaths of the mother and infant caused by disorders of pregnancy has been steadily decreasing in the past decade. Though this is true, vital statistics for the United States for the year 1947 revealed that there were 23.7 stillbirths per 1000 live births, that prematurity was the most serious cause of infant mortality, and that congenital malformations and congenital debility of unknown origin were frequently observed (U. S. Public Health Service, 1949). The infant mortality rate for the United States as a whole was 32.2 per 1000 live births,

the lowest rate in history. This average figure represents a rate of 30.1 for the white population, 47.7 for negroes, and 65.7 for non-white infants including the American Indian, Chinese, and Japanese.

While the studies by Burke and her associates as well as those of many others have been especially valuable since they have been conducted on human subjects, they are only a beginning to a complete understanding of the importance of good nutrition during pregnancy. To date little is known about the specific factors which prevent the various disorders. Likewise, we have very little information concerning the quantitative needs of nutrients at different stages of pregnancy.

Animal experimentation, conducted under more rigidly controlled conditions has also stimulated a keen interest in the importance of good nutrition during pregnancy and in the possibility that certain of the B-vitamins may be important in the prevention of abnormalities which are occasionally observed in infants. The extensive studies of Warkany and his co-workers (1942, 1943, 1944) have shown that riboflavin is a vital factor in the normal development of the embryo. When inadequate amounts of this vitamin were consumed by the mother, malformations of the palate, syndactylism, club-feet, and shortening of certain bones became apparent in the young. Recently two other publications have suggested

that malformations of the newborn are due to deprivation of certain B-vitamins. Boisselot (1948) has observed defective fetuses as a result of too little pantothenic acid in the maternal diet. O'Dell and Hogan (1950) have reported a high incidence of hydrocephalics among young rats born to animals fed a folic acid inhibitor. Withdrawal of biotin (Kennedy and Palmer, 1945), pantothenic acid (Nelson and Evans, 1946), and B_{12} (Emerson et al., 1949; Bear, 1950) likewise have been found to induce disorders of pregnancy.

With these challenging observations in mind, plans were made for a rather extensive investigation of the importance of the B-vitamins during pregnancy. While these experiments were to be limited to reproduction in the albino rat for the present, it was hoped that any special stress periods observed for the rat would be considered critically for human reproduction. Eventually deficiency studies of the B-vitamins were anticipated to determine whether inadequate amounts of these factors might bring to light additional malformations in the offspring which might aid in explaining defects observed in infants. Such information should ultimately lead to their prevention.

As a beginning to this larger program, it was believed advisable to study the normal stock animal during pregnancy and to observe both the rate of prenatal development of the young and the rate of deposition of certain vitamins in

placental and fetal tissues at intervals during the gestation period. This information should reflect the vitamin needs of the mother during pregnancy and should make us aware of fluctuations in these needs at different intervals of pregnancy.

The present study has been divided into three major sections. Part I includes a detailed review of the literature describing the embryonic development of the rat. Data are presented on the prenatal growth of normally developing young of stock animals produced in the Nutrition Laboratory of the Foods and Nutrition Department. Part II of the study includes information on the occurrence of thiamine in fetal tissues and of the thiamine stores of the female throughout pregnancy. A limited number of observations are also presented on the urinary excretion of thiamine of pregnant and non-pregnant littermates. These findings have been included since they offer an additional means of estimating the thiamine requirement during reproduction. Part III of this investigation deals with the deposition of riboflavin in maternal and fetal tissues. This section likewise includes data on the excretion of riboflavin by the kidney.

PART I

EMBRYOLOGICAL DEVELOPMENT OF THE RAT

Excellent descriptions of certain phases of the prenatal development of the rat may be obtained through the work of Sobotta and Burckhard (1911), Kirkham and Burr (1913), Fraser (1882), Selenka (1884), Cristiana (1892), Duval (1891), Robinson (1892, 1904), Widakowich (1907, 1911), Huber (1915), Grosser (1909, 1927), and Long and Burlingame (1938). Due to the magnitude of this problem most writers have limited their observations to a specific period of gestation. A number of papers describe the development of a single organ; still others have emphasized the effect of surgery on the mother on the developing fetus. The absence of a detailed consecutive account of the daily prenatal development of the rat from the time of ovulation to parturition has made it seem advisable to bring together such information and to present it in a form which will be useful to the student of nutrition. The writer wishes to arouse the interest of future students of nutrition in the endless opportunities for research on the influence of the composition of the diet of the mother on the development of her young.

The embryonic development of the rat is similar to that of all mammals. There are, however, outstanding differences, such as the inversion of germ layers which make it unique.

Mammalian embryology varies also in the relative amount of fetal tissue which is produced and the length of the period of gestation. In the case of the rat the mass of fetal tissue as well as its rate of formation is comparatively high.

Some method of stating the age of the rat embryo is necessary in order to discuss its development. This has been done in (1) general terms, such as the segmentation stage, blastocyst stage, limb-bud stage, etc.; (2) by the crown-rump length; (3) by the number of somites; or (4) by the day of gestation. In early stages of embryonic life it is impossible to use the crown-rump measure or the somite number. In later stages the development is very rapid and the somite number is most satisfactory. The method most commonly used is the day of gestation, which is reckoned from the time of insemination. It is often desirable to use a combination of these methods.

The age of the embryo cannot be precisely determined for the exact time of fertilization is not known. Keen (1947) states that most workers believe that fertilization does not take place until 9 to 12 hours after insemination. The fact that Sobotta and Burckhard (quoted by Huber) found live sperm in the genital tract 10 to 12 hours after insemination, makes this time interval a possibility. The ova may be at various stages of maturation when ovulation occurs, thus influencing the speed with which they mature upon fertilization.

Therefore embryos in the same litter may be in different stages of development. Goss (1938) observed littermates with no heart tissue formed and others with the heart developed in a saccular organ and contracting. Long and Burlingame found embryos in the first and sixth somite stage in a single litter. There is also more or less variation in the stage of development between the embryos of different litters even though their age is based on the time of insemination. These points are emphasized so that the reader will keep in mind that the period of gestation used in the following statements as well as the amount and type of development occurring per stage are at best approximations. Considerable care has been taken, however, to have the intervals as accurate as possible.

The normal gestation period of the albino rat is between 21 and 23 days. This period has been divided into several stages. Venable (1939) has classified it into four stages: (1) from fertilization to implantation, (2) from the sixth to eleventh day, (3) from the 11th to the 15th day, and (4) from the 15th day to term. Keen has divided pregnancy into three periods of seven days each, and Nicholas (1949) into three which are subdivided into 40 stages. Keen has interestingly correlated the rat and the human gestation periods. The first seven days of pregnancy in the rat approximate the first four weeks in the human; the second seven days, the fifth to the seventh week in the human; and the third seven

days to the period in the human beyond the seventh week, i.e., when the embryo begins to enter the fetal stage.

Ovulation, Maturation, and Fertilization

The subjects of ovulation, maturation, and fertilization will be discussed only briefly since excellent accounts of these processes are given in detail by Sobotta and Burckhard (1911), Long (1912), and Kirkham and Burr (1913). The material cited in this section on the development of the embryo through the blastocyst stage has been taken, in the main, from Huber's account.

The sex cell or gamete of the female develops in the ovary. This cell has many of the constituents which are contained in all animal cells, but in addition it contains an important material in its nucleus by which individual inheritance takes place, the chromosomal substance of the nucleus. Every species has a definite number of chromosomes which are always present in pairs. In the rat the number is sixteen. As the sex cell matures it undergoes two divisions. At each time two daughter cells are formed. One of these cells, called a polar body, degenerates rapidly since it receives little of the stored food material.

In the first division the pairs of chromosomes move apart. Each daughter cell therefore receives only one of each pair of chromosomes. The daughter cell which received the stored

food material divides again. In this division the chromosomes split longitudinally. The ovum is then in the pronucleus or reduced nuclear stage.

A similar reduction takes place in the maturation of spermatozoa. Two cell divisions occur, but instead of the formation of polar bodies, all cells mature. As a result of maturation the ovum and sperminum contain half of the required number of chromosomes and both are in the pronuclear stage. When they unite later the species number of sixteen pairs of chromosomes is obtained.

In the rat as in many mammals, several ova mature simultaneously. The ova are clumped together as they come from the ovary; each is surrounded by a limiting membrane known as the oolemma. The portion of the oviduct nearest the ovary is funnel-shaped, and it is in this part that the ova first lodge and here that fertilization takes place.

Attempts have been made to learn how rapidly spermatozoa traverse the uterus. Warren (1938) and also Rossman (1937) found spermatozoa throughout the uterine cornua a few minutes after insemination. Blandau and Odor (1949) observed spermatozoa in the ovarian portion of the oviducts of 86 per cent of their animals 45 minutes after insemination. The speed of travel has been thought to depend upon the muscular action of the cornua and the uterine fluid.

Spermatozoa which reach the oviduct fertilize the ova.

The head, middle section, and even a part of the tail may pierce the cell wall of the ovum and enter the cytoplasm. According to Kirkham and Burr, fertilization may occur when the ovum is undergoing the second maturation division. (Figure 1) (Kirkham and Burr, 1913)

Pronuclear to Blastocyst Stage

The fertilized ovum in the pronuclear stage contains two pronuclei, each of which is enclosed in a membrane. The smaller of the two is considered the male pronucleus (Figure 2). Huber's measurements of the ovum indicate that at this stage it is 70 by 62 microns. The pronuclear stage lasts at least 24 hours. Huber sacrificed pregnant rats 24 hours after insemination and found all the ova in the pronuclear stage. (Huber, 1915)

The oviducts of the rat are 2.5 to 3.0 centimeters in length. They are located on the distal end of the uterine horns. Twenty-four hours after insemination the ova have advanced about one-fourth of the distance of the oviduct. Their passage has been observed by Odor and Blandau (1947) to be due to muscular activity of the organ and the presence of cilia. Huber described the oviduct as containing 10 major folds and a mucosa with many loops.

After the fusion of the pronuclei, first segmentation

occurs. The cells or blastomeres formed are about the same size and structure as the ovum. Huber describes them as being oval, having a membrane around the nucleus, and possessing chromatin granules on the linin network and a chromatoid nucleolus (Figure 2). The cytoplasma was observed to be granular. Huber found the two-cell stage lasted from the middle of the second day to the end of the third day following insemination. During this time the ova had moved approximately 1.4 centimeters from the ampullar end of the oviduct, approximately one-half its length.

The two blastomeres of the first segmentation do not divide at the same time so a three-cell stage is formed. By the end of the third day both blastomeres have divided and the four-cell stage is reached. Ova in this stage are found near the last loop of the oviduct which leads to the uterine cornua. The distance is approximately 2.25 centimeters from the fimbriated end.

The eight-cell stage was found by Huber in the oviduct of a rat killed three days and 17 hours after insemination. Six ova obtained from this animal were still in the eight-cell stage, and one was in the 11-cell stage. By the end of the fourth day the segmentating ova contain 12 to 16 blastomeres. This mass of cells resembles a mulberry and is called the morula stage (Figure 3). At this stage the ova pass into the uterine horn. No ova are found in the oviduct after the

beginning of the fifth day.

The uterus of the rat is bicornate, or two-horned. It is made up of two layers, the myometrium or muscular layer and the mucosa or endometrium. The muscular layer in turn consists of a double layer of muscles, one lying in a longitudinal direction and the other being circular. These two groups of muscles allow very vigorous contraction of the uterus. Between the muscular layers is a vascular zone.

Shortly before ova leave the ovary, cell proliferation occurs in the myometrium and endometrium. The mucosal glands of the endometrium increase in size due to the growth of their epithelium. The cells change from cuboidal to columnar in shape. Some change takes place in the lining epithelium as well as in the glands. The subepithelial connective tissue also shows hyperplasia and an increase in blood supply including new growth of blood vessels. Reynolds (1939) states that while the uterus is highly active at the time of oestrus it becomes quiescent before the ova reach it. According to Allen (1931), the epithelial cells produce a secretion which appears to nourish the embryo during the first days of implantation (Block, 1939).

During the first half of the fifth day the ova progress along the uterine horn, spacing themselves in the position they hold when they become attached to the mucosa. They lie free in the lumen of the uterus, but are lodged in the valleys

between the folds of the mucosa.

It would be interesting to know what factors are responsible for the passage of the ova along the uterine horn and what accounts for the fairly regular spacing they assume. Widakowitch, as quoted by Huber, observed short cilia in the uterine lining. These cilia were not found by Huber, although he suggests that they might be present for only a short period of time. Huber cites Mandl as having found cilia in other animals than the rat. Mossmann (1937) suggests that some type of physiological relation exists between the maternal mucosa and the embryo which renders the immediate region about the implantation site refractory to any other embryo, although he has no experimental proof for this theory. Block (1939), working with mouse eggs, believes there is a chemotactic attraction of the egg to the uterine secretion. The areas of strong secretion attract the blastocyst causing their implantation. It has been observed that fertilized ova may migrate from one uterine horn through the common cavity of the uterus into the opposite cornu and become implanted there.

The cells of the morula continue to divide. They not only increase in number but begin to show some differentiation. At the 24- to 30-cell stage the cells at one side or pole begin to separate from the others. This arrangement forms a central cavity. The embryo is then in the early

blastula stage, which occurs in the latter part of the fifth day following insemination (Figure 4).

Throughout the sixth day of gestation the blastocyst increases rapidly in size due mainly to an enlarging of the blastocele (Figure 5). The cluster of cells at one side become increasingly prominent. This section, known as the germinal disc, will eventually become the body of the embryo. With the continued thickening of the germinal disc, cells grow out as well as into the cavity of the blastocyst. The former become the ectoplacental cone and the latter the ectodermal node and the cells which surround the yolk-sac (Figures 6 and 7).

The blastocysts are still not attached to the uterine mucosa at this stage although the latter does show a reaction to their presence other than the histological one mentioned above. There are swellings on the uterine tube caused by a thickening of the mucosa at the position of each ova. Decidual crypts are forming on the antimesometrial side. These are bell-shaped at first and open to the uterine lumen. The sixth day after insemination the embryo begins its implantation in the crypt.

Fig. 1. Tube egg showing the first polar body at the top, the second in the process of formation at lower left, sperm head at right with portion of tail. Follicle cells outside. From Kirkham and Burr (1913, fig. 14) X 500.

Fig. 2. Tubal ova. Upper figure taken from the rat 24 hours after insemination. Ovum is in the pronuclear stage. Lower figure taken from the rat 2 days after insemination. Thin oolemma showing two-cell stage. From Huber (1915, fig. 1) X 200.

Fig. 3. Section of morula stage of the rat, 16-cell stage. From Huber (1915, fig. 19) X 200.

Fig. 4. Section of early stage of the blastodermic vesicle. Taken 5 days after insemination. From Huber (1915, fig. 22) X 200.

Fig. 5. Section of blastocyst taken 6 days after insemination. From Huber (1915, fig. 23) X 200.

Fig. 6. Section of blastocyst showing early stage of entypy of germ disc. Note the ectodermal node in the cell mass, the visceral layer of entoderm seen around lower portion of it and the ectoplacental cone at top of figure. From Huber (1915, fig. 24) X 200.

Fig. 7. Section of blastocyst, more advanced stage. From Huber (1915, fig. 24) X 200.

Fig. 8. Longitudinal section of egg-cylinder showing proamniotic cavities. From Huber (1915, fig. 27) X 200.

Fig. 9. Longitudinal section of egg-cylinder showing fusion of the proamniotic cavities. From Huber (1915, fig. 27) X 200.

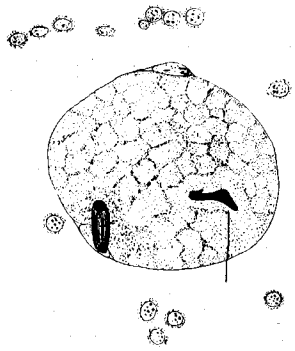
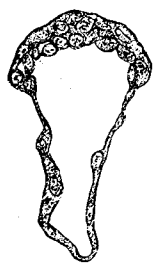


Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.



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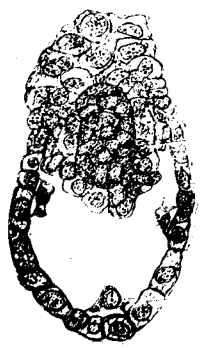
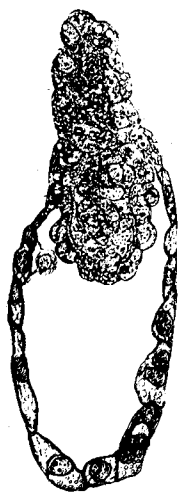


Fig. 6.



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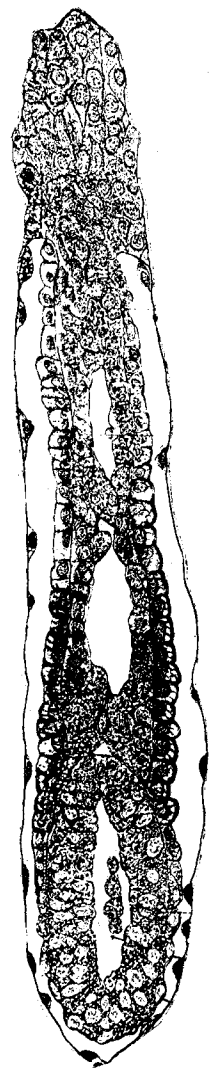

Fig. 8.

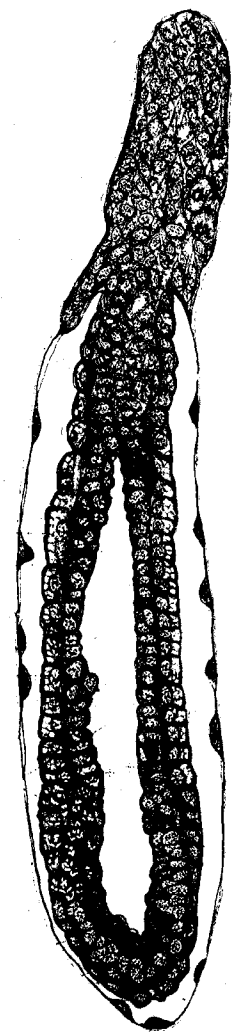

Fig. 9.

Implantation to the Tenth Day

Implantation in the rat is different from that of many mammals for there is a shift of attachment from the antimesometrial to the mesometrial position with both co-existing for several days (Figure 10). Alden (1945) showed that the antimetrial region first attracts the blastocyst when he surgically inverted the uterus of the rat and found that implantation still occurred on the antimesometrial side. A number of differences have been observed between the two regions. Nicholas (1947) stated that there are a greater number of uterine glands in the antimesometrial region, the mucosa is less dense, and the epithelium becomes pseudostratified while the mesometrial region is still in a columnar form. Krehbiel (1937) and Long and Evans (1922) found that the formation of decidua could be induced by mechanical, chemical, electrical or other forms of stimulation. The mere presence of the embryo seemed to cause these developments in the uterus.

The circulatory system on the mesometrial portion consists of rather large vessels while that on the antimesometrial side includes a capillary network. The latter permits a greater amount of potentially available food material.

During early pregnancy Alden (1947) found that fat accumulated in the epithelial cells throughout the length of the uterus. Since fat was not present at the site of

Fig. 10. These diagrams show stages of implantation. The lower portion of the figure is the mesometrial side of the uterus.

Drawing 1 shows the progress of implantation on the sixth day after insemination; drawing 2, the seventh day; 3, the eighth day; 4, the ninth day; and 5, the tenth day.

In drawings 3, 4, and 5 blood, formed from the erosion of the subepithelial decidual vessels, is apparent. This bleeding is detectable in the vagina on approximately the 11th day after insemination. From Venable (1939, fig. 1-5).

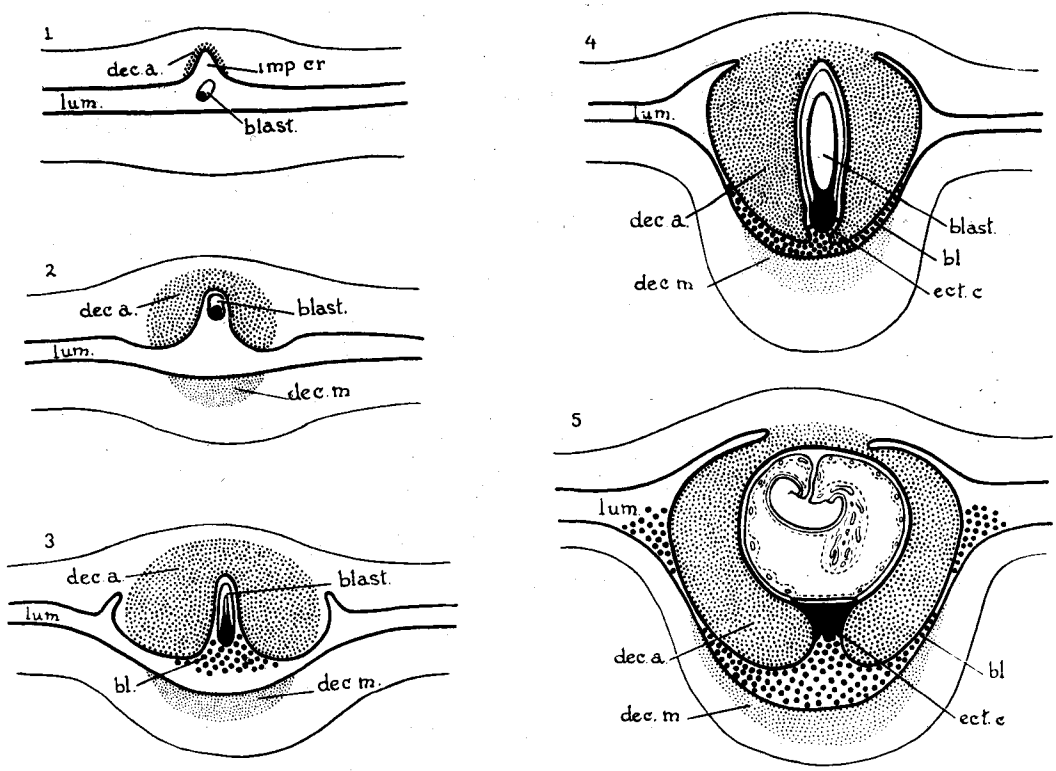

Fig. 10.

ABBREVIATIONS

bl., blood
blast., blastocyst
dec. a., antimesometrial decidua
dec. m., mesometrial decidua
ect. c., ectoplacental cone
imp. cr., implantation crypt
lum., lumen

implantation, Alden concluded that the blastocyst had used it. Wislock and Dempsey (1945) found iron, glycogen, lipoids, alkaline and acid phosphates, nuclear protein and calcium in the endometrium of rats. Krehbiel (1937) also found the anti-mesometrial portion of the rat uterus rich in lipoids and by the eighth day of pregnancy the mesometrial cells were filled with glycogen. These facts illustrate a difference in the kind of preparation and in the rate of preparation between the mesometrial and antimesometrial regions.

During the seventh day the cell mass, which extends into the blastocele or central cavity, shows further differentiation. In it develops a group of cells which stain more deeply than the other cells. It is called the ectodermal node, and in the future it will become the ectoderm of the animal (Figures 6 and 7). This group of compact cells growing into the blastocele becomes the egg-plug or egg-cylinder. It is covered by a single layer of cells which will become the entodermic cells of the animal. The decidual crypt in which the seven-day blastocyst is lodged is still open to the lumen of the uterus and contains its epithelial lining. The blastocyst is so lodged that the thicker portion or germ disc is adjacent to the mesometrial border.

In the eighth day of gestation the blastocyst increases in length. The ectodermal node, which is in the cell mass first formed in the ectoplacental cone is pushed farther into

the blastocele. This change of position is caused by an increase of cells at the base of the ectoplacental cone. The group of cells causing the antimesometrial growth of the ectodermal node is called extraembryonic ectoderm (Figure 8).

The cylindrical mass of cells consisting of the (1) ectodermal node, (2) the extraembryonic ectoderm, and (3) the layer of entodermal cells is known as the egg-cylinder. This section elongates, due partly to increase of cells and partly to the formation of an internal cavity. The cells of the ectodermal node become arranged in a single layer and a central cavity is formed. Soon two other cavities develop due to a rearrangement of cells in the extraembryonic ectoderm (Figure 8). In a few hours these merge into one cavity (Figure 9).

During this period the ectoplacental cone has grown toward the lumen of the uterus. The decidual crypts are deeper and narrower but still open (Figure 10). The cells of the ectoplacental cone and the thin membrane of the blastocyst (the trophoblast) have a phagocytic action for maternal blood cells in the decidua of the mucosa. Therefore, during this period the blastocyst is capable of obtaining maternal hemoglobin, which accelerates its growth considerably.

The appearance of entodermal cells covering the ectodermal cells is not common in mammalian embryos. In the majority of the class Mammalia the entoderm is formed from

cells which are pushed out from the inner cell mass; in these embryos the entodermal cells form a complete layer inside the ectodermic cells.

In the embryo of the rat, however, the cells which will become the entodermal tissues are outside the ectoderm. They surround the egg-cylinder described above. This arrangement of germ layers is known as the inversion of germ layers, or entypy.

It was mentioned above that maternal blood is obtained by the blastocyst on the eighth day. Venable (1939) found extravascular blood on the eighth day in the implantation crypt between the wall of the crypt and the extra-embryonic ectoderm. The amount of blood present was small. He noted two sources of intra-uterine bleeding. Erosion of decidual vessels by the phagocytic action of the ectoplacental cone is responsible for one source of blood, and intra-uterine bleeding due to the blood sinuses of the giant cell area is the second source.

During the ninth day of gestation the embryo completes the development of the amnion, a membrane which surrounds it and marks it off from the cavity into which the allantois will grow (Figures 11 and 12). Nicholas and Rudneck (1934) found the embryo of this age in the antimesometrial third of the blastocyst; the middle third is the false amnion cavity; and the mesometrial third contains the ectoplacental cone.

Fig. 11. Blastocyst at the end of the ninth day. Schematized from Grosser (1927, fig. 131). From Everett (1935, fig. 3) X 30.

EC . . . ectoplacental cone
CZ . . . central zone
E CV . . ectoplacental cavity
VE . . . vitelline epithelium
EX . . . exocoele
A . . . allantois
AM C . . amnion cavity
RM . . . Reichert's membrane

Fig. 12. Early 10-day stage. Ectoplacental cavity nearly obliterated. The allantoic stalk growing toward the base of the cone. From Everett (1935, fig. 4) X 30.

MBL . . maternal blood lacuna
LM . . . lamina
YS C . . yolk sac cavity

Fig. 13. Showing neural folds forming the forebrain, midbrain, and hindbrain. Neural plate and somites are posterior to the brain divisions. From Henneberg (1937, fig. 28) X 25.

Fig. 14. Schematized longitudinal sections illustrating the formation of the head fold and the fore-gut, in which ectoderm is shown as solid, entoderm in shading, and mesoderm in stipple. (A) shows early stage in formation of head fold. (B) shows a later stage with the head fold grown farther forward, increasing the length of the fore-gut. From Wieman (1930, fig. 58).

ap . . . anterior intestinal portal
fg . . . fore-gut
hf . . . head fold
mp . . . medullary plate
op . . . oral plate

Fig. 15. Photomicrograph of section of 9-day 16-hour rat embryo, through lateral heart promordia. From Goss (1937, fig. 6) X 130.

Fig. 16. Sagittal section, showing the first somite undergoing differentiation. From Butcher (1929, fig. 23) X 104.

S.m. . . somite
P.c. . . pericardial cavity

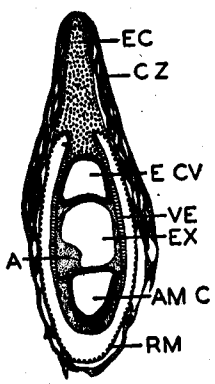

Fig. 11.

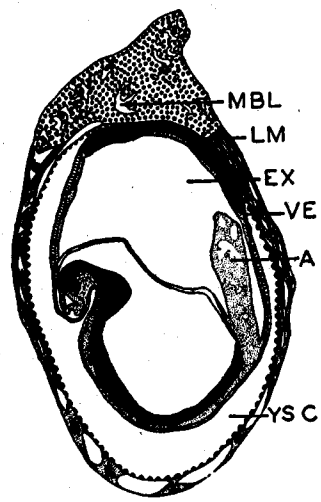

Fig. 12.

Fig. 13.

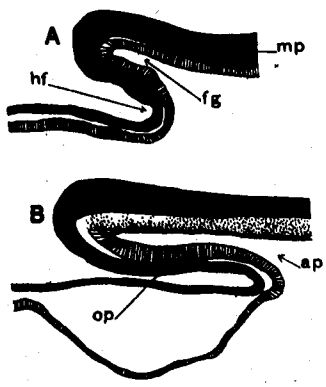

Fig. 14.

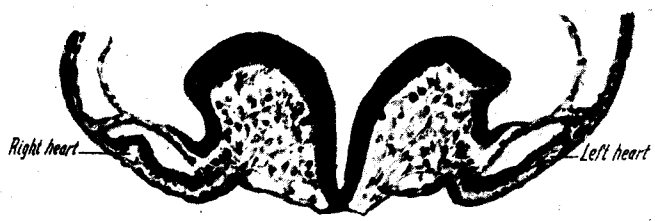

Fig. 15.

Fig. 16.

The decidua with the ectoplacental cone of the blastocyst push out mesometrically into the uterus, filling the decidual crypt, and almost filling the lumen of the uterus, at each implantation site. At this stage it is difficult to distinguish the ectoplacental cone from the maternal decidua.

In the blastocyst there is a heightened rate of cell proliferation, with an increase in thickness. A portion of the blastocyst becomes folded or indented. This portion is known as the primitive streak, or the growth center. It develops at the junction of the primary embryonic and extra-embryonic portion of the blastocyst. The primitive streak establishes the longitudinal axis of the embryo. In the rat the structure is U-shaped for it extends around the cylinder (Figure 12). At the anterior end of the primitive streak is an area called Hensen's node from which the notochord originates. This cord is comprised of a rod-shaped mass of cells extending the length of the body, ventral to the central nervous system. The notochord indicates the future location of the vertibral column.

From the primitive streak mesodermic cells proliferate between the entoderm and ectoderm of the primary and extra-embryonic portion of the blastocyst. They increase rapidly and soon establish a layer of cells which extend out peripherally. The lateral portions split into two layers, which form the somatic and splachnic mesoderm.

During the time the mesoderm is first forming, a portion of the ectoderm anterior to Hensen's node becomes thicker than the rest. This portion is called the neural plate. It is the first embryonic rudiment of a permanent organ to be developed; as the neural plate is formed it becomes folded, the middle part is depressed, and the sides are elevated. In this way a groove is formed longitudinally with folds laterally. The folds are known as neural folds. The neural folds also bend anteriorly, forming the head folds of the embryo. With the forming of these structures the foregut is initiated (Figure 14).

One of the outstanding developments of the ninth day is that of the heart. This organ originates in the mesoderm. At first it is a paired structure, the two sections lying separated on either side of the axis of the embryo. Sagittal sections of the heart region of the blastocyst show the primordial heart as a space or coelome in the splachnic mesoderm (Figure 15). The heart is comprised of two layers. The inner layer or endocardium is at first just irregular cells lying between the splanchnic mesoderm and the entoderm. These cells organize and form a lumen. The outer layer, the epicardium will form the muscular layer of the heart. When the lateral regions of the embryo fold together, the two portions of the heart, or two hearts, unite.

The heart becomes a saccular organ at the same age that

the head-folds form. It does not develop into a four-chambered organ until much later, although it begins to function by the ninth or tenth day. As the head folds develop they bend forward until they touch the ectoderm covering the primitive heart (Figure 16).

The first contraction of the heart, according to Goss (1938) occurs at approximately nine and one-half days. Of forty embryos studied by this investigator, contraction was observed to start in the left heart first in all but two cases. Contraction in the right heart began about two hours later. Goss describes the first contraction as a twitching of three or four cells in the ventricular myocardium, near the primitive atrium and ventricle. The contractions were wave-like in character at first, becoming stronger and subsequently more frequent. During this early period the rate varied from 37 to 42 contractions per minute; the right heart rhythm was considerably slower than the left. After the hearts united the left side regulated the rhythm. It is interesting that circulation of blood did not begin until approximately 12 hours after the first contraction.

Burlingame and Long (1939) found that in some cases the heart develops as a single organ rather than as a bilobed structure; i.e., the heart does not always develop in exactly the same way. They observed circulation in their embryos when the young were in the eighth somite stage.

During the ninth day, the allantois starts its growth out from the primitive streak into the ectoplacental cavity (Figure 12). It resembles a bulb and is composed of mesoderm.

In the rat there are two placental attachments, the ectoplacental and the yolk-sac. Everett (1935) found that they existed concurrently throughout the latter half of pregnancy. The yolk-sac develops first. The allantois placenta does not become established until approximately the 12th day. Workers have disagreed as to the relative importance of the two placentas. Everett suggests that the yolk-sac is an organ of exchange and that it is not secondary to the allantois placenta. Noer and Mossman (1947) found that the allantoic placenta was vital for life although the true function of the yolk-sac placenta was less certain.

During the 9th, 10th, and 11th days, Nicholas and Rudneck (1934) observed that the decidua in the yolk-sac region thins, the epithelium thickens and extends completely over the embryo.

By the end of the ninth day the embryo has a well developed functioning primitive streak. The head folds and allantois have started to form. The embryo is separated from the ectoplacental cavity by the amnion. The three germ layers are initiated. The first to form, which is the ectoderm, gives rise to the nervous system. The entoderm forms the lining of the digestive tract, and the mesoderm contributes to the main skeleton, the muscles, and the circulatory organs.

Tenth Day (1 to 19 Somites)

The formation of somites is very rapid in the rat. In fact, according to Butcher (1929) several somites may form at approximately the same time. They develop from the mesoderm which is observed to thicken and form somatic plates which in turn segment into somites (Figures 13 and 17). The cells of the various somites do not have a common fate. In each somite the cells take on a radial arrangement with a lumen or myocele in the center. The cells in the dorso-mesial part will form the skeletal muscle which develops at that level of the body; they are known as myotome. Those cells in the ventro-lateral portion will migrate out and contribute to the formation of connective tissue under the epidermis. These are called dermatome. Some of them may also form muscle. The third region is called sclerotome; this section consists of cells which migrate toward the neural tube and notochord, forming the vertibrae (Figure 18).

Species vary widely in the number of somites which they form. In the rat there are 65 pairs, all of which are formed by the 16th day of gestation.

The development of the brain progresses rapidly during the tenth day. The neural tube is formed from the neural plate. The anterior part enlarges and soon three regions are differentiated. These are the forebrain, midbrain, and hind

Fig. 17. Sagittal section of an embryo showing the migration of the sclerotome in the second somite and the relation of the first intersegmental vessel. From Butcher (1929, fig. 35) X 104.
F.g. . . . fore-gut
S_2 . . . somite

Fig. 18. Transverse section showing differentiation of somites. From Patten (1946, fig. 40). X 120.

Fig. 19. Schematic drawing of sagittal section of an embryo showing the anterior and posterior intestinal portals with the wide mid-gut. From Patten (1946, fig. 70a).

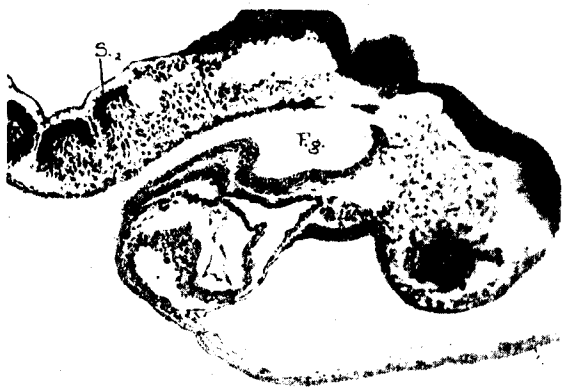

Fig. 17.

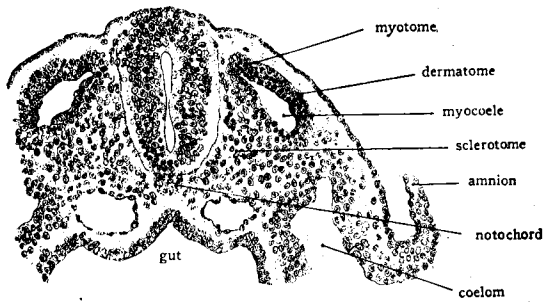

Fig. 18.

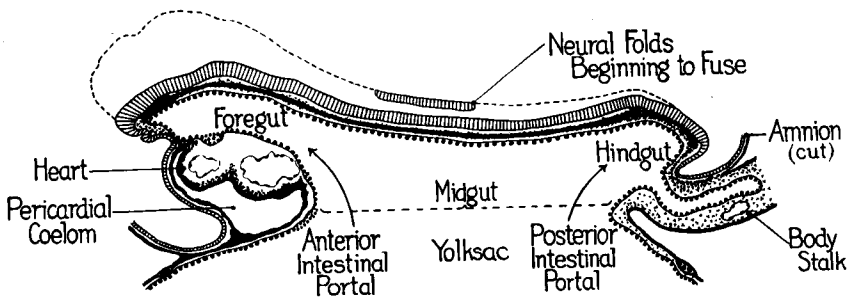

Fig. 19.

brain, the prosencephalon, mesencephalon, and rhombencephalon, respectively.

Besides the brain the neural tube develops neuromeres. Of the seven distinguishable in the rat (Adelmann, 1925) the first corresponds to the hind brain and the others become a part of the spinal cord. All mammals, including the rat, have 12 cranial nerve ganglia. Their original location has been correlated with the neuromeres by Adelmann. The optic vesicles protrude as lateral bulges on the anterior part of the head. These are easily recognized in the embryo ten days of age.

It has been mentioned that the anterior intestinal portal was formed at the time of the forward growth of the head folds. By the 6th or 7th somite stage the posterior intestinal portal is formed. These portals are both entodermically lined pockets which end blindly anteriorly and posteriorly. Later, depressions occur on the surface of the body and sink in to meet the gut, thus forming the oral and anal openings. The primitive gut has a wide mid-portion which gradually narrows as development increases (Figure 19).

The heart protrusion becomes increasingly more prominent during the 10th day. Between it and the optic protuberances, i.e., in the neck region, a series of elevations appear. These are the gill arches or brachial arches. The most anterior one, known as the manibular arch, is situated close

to the future mouth (Figures 20 and 21). Posterior to it are the hyoid arch and two unnamed post-oral arches. These arches are composed of all three germ layers. The mandibular arch will give rise to the upper and lower jaws while the other arches will become incorporated into the neck.

Concurrent with the formation of the gill arches the foregut becomes compressed dorso-ventrically, pushing it out to the side. The pouches formed are homologous with the furrows between the brachial arches. In water-living ancestral animals the gill clefts were gill slits.

The mandibular arch develops by the 8th or 9th somite stage, and is soon followed by the hyoid arch. At the 10th somite stage a depression above the furrow between the arches appears. This is the site of the inner ear. This depression deepens, and by the 16th or 17th somite stage it has formed a pit.

The vascular channels leading from the heart are formed by groups of mesodermal cells which aggregate along the developing artery or vein. These cells become hollowed out into tubes. Those arising from the heart run cephalically beneath the pharynx, then bend to the side and back over the pharyngeal walls, thus forming what is known as the aortic arches. The channels turn and extend the length of the embryo, thus forming the dorsal aortas.

The first aortic arch is located in the mandibular arch.

Fig. 20. Embryo of 21 somites. Mandibular and hyoid arches shown here. Note location of ear vesicle and anterior limb bud. From Long and Burlingame (1938, fig. 27a) X 20.

Fig. 21. Embryo of 34 somites. Formation of the maxillary process. Hyoid arch commencing to cover third arch. From Long and Burlingame (1938, fig. 29) X 10.

Fig. 22. Face of embryo of 34 somites. Mandibular arches beginning to fuse, forming the lower jaw. From Long and Burlingame (1938, fig. 29a) X 10.

Fig. 23. Face of embryo showing mandibular arch. Medial nasal processes are fusing. Maxillary processes will form upper jaw. From Long and Burlingame (1938, fig. 31a) X 5.

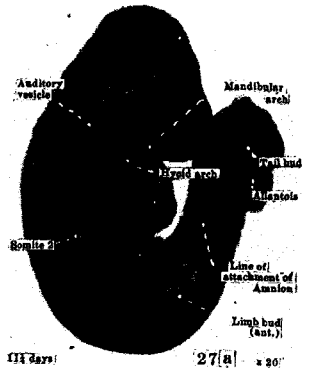

Fig. 20.

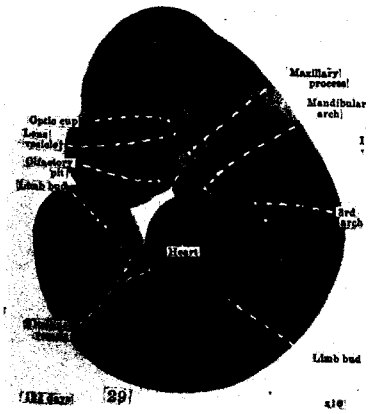

Fig. 21.

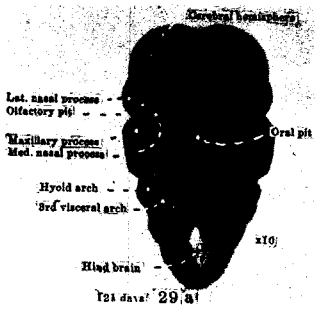

Fig. 22.

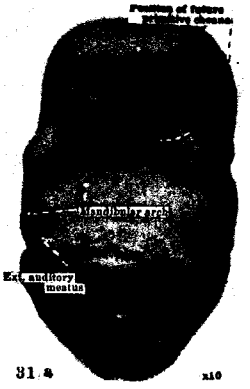

Fig. 23.

Henneberg (1937) found it in the brown rat at about the 4th somite stage. The second aortic arch forms at the 8th to 9th somite stage. By the 19th somite stage both the first and second aortic arches are well established.

In the brown rat, according to Henneberg, the liver bud begins to develop at about the 8th to 9th somite stage. It develops as an outgrowth of the gut. By the 19th somite stage, or the end of the 10th day, the liver has enlarged and is found to contain trabiculae.

The thyroid also begins to develop during the 10th day. It begins as an endodermal bud located mid-way on the floor of the pharynx. The primitive kidney, known also as the Wolffian body, is first seen at about the 12th somite stage.

When the embryo is first being formed the dorsal surface is concave. The midgut is at first wide and flat. Later it folds together forming a narrow crevice. As growth continues the distance between the foregut and the hind gut becomes narrower. The allantois grows out from the hind gut and attaches itself to the ectoplacental cone. It eventually forms the placental attachments. The attachment of the yolk sac and that of the allantois is in opposite directions. This difference in direction causes a twisting of the embryo which has turned to the left. The tail is drawn toward the right due to the yolk sac attachment. The embryo eventually becomes turned so that its dorsal surface is convex instead of concave.

Eleventh Day (20 to 26 Somites)

The cerebral hemispheres which are just distinguishable at the 16th somite stage enlarge and are very pronounced when the rat embryo possesses 26 somites. Adelmann (1925) found the diencephalon and telencephalon well marked at this time. The neural plate is closed except in the caudal portion where the allantois is attached.

The heart bulb becomes more conspicuous at this age. In the brown rat, Henneberg noted trabiculae beginning to form in the ventricles of the heart by the 11th day and three aortic arches were well developed. The optic visicles are seemingly pushed back by the enlarging cerebral hemispheres toward the region from which the maxillary processes soon appear. The auditory pit which has a minute opening at 21 somites is closed by the 24-somite stage. At the 26th to 30th somite stage the olfactory pits are barely distinguishable.

The maxillary process is first seen at 21 somites (Figure 21). It forms from the upper portion of the mandibular arch and grows toward the eye. The hyoid arch and the third arch are plainly distinguishable at this stage. The optic swellings now appear as shallow pits. These will deepen and later will be cut off. These changes occur at approximately 34 somites or the 13th day. From that time the position of the eye is known by the developing eyelids.

The anterior limb buds appear as swellings on the side of the body at about the location of the 6th to 10th somites. These are first observed at the time of the development of the 21st somite (Figure 20).

The pituitary gland (hypophysis) begins to form in the brown rat at about the 23rd somite stage. It originates from two primordial parts, one known as Rathke's pocket and the other, the infundibular process. Rathke's pocket consists of ectoderm which lines the oral cavity while the infundibular process, arising in the floor of the diencephalon, is ectoderm which originated from the neural tube. These two parts form the anterior and posterior pituitary gland.

During the 11th day, the pancreas begins to develop. It starts as a bud on the dorsal wall of the duodenum. On the 13th day another bud appears on the ventral wall. These are known as dorsal and ventral pancreatic buds. During the 15th day they fuse.

From the 11th day to the 15th the antimesometrial decidua of the uterus gradually get thinner. Some blood is released in this process. It cannot escape, however, for the embryo fills the lumen. Venable (1939) suggested that this material is resorbed, since he observed it between the embryos of pregnant rats sacrificed on the 12th day. The blood which goes into the vagina comes from the lowest implantation site in each uterine horn. This change accounts

for the red blood cells found in the vagina when the contents are examined on the 11th or 12th day after insemination. Their presence is often taken as an indication of pregnancy.

Twelfth Day (27 to 33 Somites)

One of the most noticeable developments of this period is in the region of the future face. The olfactory pit which is located on the ventro-lateral region of the cerebral hemispheres is scarcely distinguishable at 26 somites. It deepens rapidly and becomes vascular. The maxillary processes which are growing forward toward the eyes contact the external nasal processes (Figures 22 and 23). The olfactory pits become narrower and smaller and draw closer together. They do not fuse, however, until the following day. Henneberg describes the brown rat of 12 days as having the telencephalon, diencephalon, mesencephalon, and isthmus of the head easily distinguishable. The fifth, seventh, and eighth ganglia are very distinct at this time. (Henneberg, 1937)

Vibrissae begin to develop on the head, and by the next day they are seen over the entire body.

At the 26th somite stage the mandibular arches are separated ventrally by a cleft. They fuse during the 12th day of gestation, forming the lower jaw. The hyoid arch begins to cover the third arch so the latter and the furrow between them become submerged. The ventral end of the first furrow

deepens and forms the external auditory passage. The external ear itself is formed from the remainder of the furrow. The inner ear, it will be recalled, began formation at the 10th somite stage. The lens vesicle deepens during this period and is cut off.

The heart protuberance is still prominent. Henneberg finds that the heart of the brown rat of this age is developing the interventricular septum which divides the left and right ventricles. The first aortic arch has disappeared, the second is reduced in size, but the third is complete and quite large.

As the intestine grows posteriorly it widens at the extreme end. The hind gut and cloaca are one. During the 12th day, in the brown rat at least, the urorectal fold begins to develop, which will separate the two.

At this stage the dorsal pancreas is found to increase in size. The liver develops numerous trabiculae.

According to Henneberg, the lung bud appears during the 28th somite stage. It is formed from the floor of the pharynx and elongates parallel to the digestive tract. Aldeman's model of the 26th somite embryo shows a lung bud present and also hepatic diverticulum.

It is interesting that the rat develops no gall bladder, although the mouse and most mammals do.

Thirteenth Day (34 to 47 Somites)

The umbilical and vitilline vessels which project from the ventral side of the embryo come much closer together due to the fact that by the 13th day the space between the foregut and hind gut has been greatly reduced. These vessels form the umbilical cord. The beginning of the umbilical hernia may be seen for the intestine forms a loop which protrudes ventrically at the naval. The head of the embryo becomes increasingly well defined and is as large as the remainder of the body. The eyelids begin to develop. Vibrissae cover the entire body.

The nostrils and maxillary processes continue to grow closer together. The lower jaw is fused. The mouth is a depression bounded by the nasal and maxillary processes and the mandibular arch. The heart is less visible than formerly due to the thickening of the body wall. The atria and ventricles of the heart are well established and the anterior endocardial cushion and interventricular septum are forming. The first and second aortic arches have disappeared while the third, fourth, and fifth arches are well developed.

Internal development of the ear shows an elongation of the endolymphatic duct. There are folds forming which will become the future semicircular canals.

The dorsal pancreas increases in size and there is a

ventrical pancreatic bud. The bronchial tubes are beginning to branch. A stomach is developing. The parathyroids and the epiglottis begin formation. The mesenchyme of the future bone is thickening preparatory to bone development.

The posterior limb buds develop at 34 somites. At this time the anterior limb buds have become longer and are paddle-like. Both pairs increase in length. The portion which will become the foot broadens, flattens, and develops five lobes. It resembles a webbed foot. During the 16th day the clefts between the digits deepen. Claws are formed and foot pads appear at an even later period. Though five digits are originally formed on the anterior feet, the first digit never becomes a claw. On the hind feet all five digits develop into claws with the middle three of the same length.

Fourteenth Day (48 to 60 Somites)

At this time the fetus has a less curved configuration. Its head is lifted somewhat. The snout is clearly distinguished from the rest of the head. The nose has increased in size but the nasal openings are reduced.

The eye lens does not have a lumen. The nasolacrimal duct develops. The interior wall of the eye cup becomes considerably thicker.

The semicircular canals of the ear become more pronounced. The cochlia of the ear is no longer a straight duct

but resembles a half turn.

The various structures of the mouth as the tongue, the palate, and the salivary glands show development.

The dorsal pancreas becomes more branched as does the lung bud. The genital ridge begins to form. Vertibrae appear.

Fifteenth Day (61 to 65 Somites)

The fetus at this age has 60 pairs of somites according to Nicholas (1949). Its motor reactions begin to function. The fetus and fetal membranes are free from the antimesometrial side of the uterus. From now on the fetus is attached by the chorio-allantoic placental to the mesometrial portion of the uterus.

The mouth is nearly closed by the 15th day. The palatine processes are growing closer together and will form the roof of the mouth. The tongue has enlarged. The eye of the fetus has a lens, cornea, and even a pigmented layer of the retina. The spleen, thymus, and bladder are forming during this stage (Long, 1938). The semicircular canals of the ear are well developed. The genital and mesonephric folds are clearly distinguishable. Muscle anlage appears on the ribs.

Henneberg reports that the pineal body is formed at this period. The dorsal and ventrical pancreas are united. The internal ear structures become well defined at this time,

although they are still cartilage.

The hernia, a large balloon-like mass pressed against the exterior of the body of the fetus, has increased in size. It contains coils of intestine covered by a thin membrane. It is first seen during the 15th day and increases in size until the 17th day when it is rapidly reduced by the withdrawal of the intestine into the peritoneal cavity.

Sixteenth Day

By the sixteenth day all the somites have formed. The eyes have well developed eyelids. An external ear has formed and the ears are open. The feet are still webbed, but the digits show considerable development, and the clefts between them are deepening.

In the brown rat the cochlia of the ear has elongated and twisted to a one and a half turn. The nasal openings are closed. The salivary glands are branching. The thyroid, parathyroid, and thymus are well developed. The anus is open and the cloaca is completely divided. Bone formation is visible in the collar bone and in the upper and lower jaws. At this stage the skeleton is still cartilage.

Seventeenth Day

The external appearance of the fetus is constantly

changing. At this time the head is smaller in proportion to the remainder of the body. The neck is more clearly defined. The hernia has reduced greatly in size. Two umbilical and two vitelline vessels pass from the sides of the hernia to the placenta and yolk-sac respectively.

Eighteenth Day

Outwardly the fetus shows only slight changes from that of the previous day. The neck region is gradually becoming thinner. The digits of the extremities are longer, and the tail lengthens. The eyes are still closed at this stage, and the ears are beginning to close.

Nineteenth Day to Term

Only minor changes are noticeable in the appearance of the fetus after the 17th day. The internal organs continue their development and the cartilage becomes more bone-like in character. The fetus increases rapidly in both length and weight.

In twenty-one days the fetus has grown from a microscopic cell to a complex animal weighing four to five grams.

We are indebted to the diligent and painstaking efforts of the anatomists and histologists for the detailed information concerning the physical development of the rat fetus.

Contributions of the biochemist and nutritionist have not been as numerous, probably due to the more recent knowledge of suitable chemical methods. Within the next decade it is expected that great strides will be made toward an understanding of the biochemical aspects of the embryology of the rat.

The Uterus

The uterus of the rat is a two-horned organ composed of two layers: (1) the myometrium or muscular layer and (2) the endometrium or mucosa. The muscular layer in turn consists of two layers, one of which is circular and the other longitudinal. Between the muscular layers is a vascular zone. The allantois growing from the embryo buries itself in the endometrium layer of the uterus. The membrane is known as the decidua. It is discarded at parturition. Mammals vary in the number of placental tissue layers between the blood of the embryo and that of the mother. It is interesting that the rat and man have the same number. The maternal tissues consist of (1) epithelial uterine lining, (2) connective tissue, and (3) the endothelium of the blood vessel wall. The fetal tissues include (1) epithelium of the chorion, (2) the connective tissue, and (3) the endothelium of the blood vessel walls. In some animals, for example the horse and pig, all six layers are present. In the goat there are five

layers, in the cat four, but in man, the rat, the rabbit, and guinea pig there are only three, the three fetal layers. The placenta containing only three layers is known as the hemochorial type because the fetal chorion epidermis comes in direct contact with the maternal hemoglobin.

The enlargement of the uterus during pregnancy is made possible by the fact that the cells of the uterus increase in number and in size. This is due to both hyperplasia and hypertrophy. These two processes do not occur at the same time during pregnancy, however. The production of new cells by mitosis takes place in early pregnancy before implantation of the blastocysts. Hyperplasia takes place throughout the whole uterus without changing the weight of the organ. These new cells are smaller than those from which they are derived, thus not increasing the weight.

Most of the enlargement of the uterus is due to an increase of the size of the new cells before the blastocysts become implanted. The muscles of the uterus as well as the lymphatic capillaries and blood vessels likewise undergo this adaptation process. There are many mechanisms by which the uterus adapts itself to the implantation and growth of the fetuses which have not been elucidated.

EXPERIMENTAL PROCEDURE

The general plan of the present study was to produce a series of pregnant rats which would be sacrificed at known intervals during the gestation period, in order that the rate of deposition of vitamins in placental and fetal tissues could be determined. The quantity of vitamins present in the normally developing tissues could then be used to reflect the minimum demands of the mother over and above those of the non-pregnant female. It was also planned that analyses should be made of the vitamin content of the hepatic and carcass tissues of the mother at stages of pregnancy since it was thought possible that the maternal vitamin stores might fluctuate from one stage of the gestation period to another.

The success of the study obviously depended upon selection of animals which could be depended upon for excellent reproductive performance.

Many records of stock rats produced in the Nutrition Laboratory of the Foods and Nutrition Department revealed a long history of satisfactory reproduction among this colony of animals. Females were known to produce good first litters, although second litters were considered slightly more uniform as to the size and numbers of young born. In the present investigation, therefore, healthy young females of this colony

were sacrificed during their second pregnancies.

Animals and Their Daily Care

Over seventy 28-day-old females representing Wistar stock inbred by brother-sister matings for 97 generations were selected for the study. The animals were housed in individual wire mesh cages equipped with elevated bottoms. Distilled water and the customary stock ration were offered ad libitum. The females were weighed once per week prior to pregnancy; during pregnancy they were weighed daily.

Animals were examined regularly to determine their age at maturity, which was judged by the opening of the vaginal orifice. Twenty-eight days following the opening of the vaginal orifice the females were mated. This procedure was followed in order to secure greater uniformity in the age of the animals at the initiation of pregnancy.

Breeding Technique and Establishment of the Time of Initiation of Pregnancy

Examinations of the vaginal contents were made daily when the animals were approximately 70 days of age. When the females were in a late proestrous or oestrus stage a littermate male was placed in the cage. Positive signs of mating included the copulation plug or the presence of sperm in the

vagina. If these signs were not observed by the time leucocytes again appeared in the vaginal smear, the male was removed until the onset of the next mating stage.

Young produced during the first pregnancies were weighed individually and were examined carefully for defects. These young were sacrificed immediately since the present study was limited to vitamin needs during reproduction and not lactation. Females were then remated as early as 24 hours after the birth of their first litters. It was particularly important that the time of insemination was established accurately during second pregnancies since it was hoped that subsequent vitamin assays of the various tissues would reflect vitamin needs at rather narrow intervals during pregnancy. Examinations of the vaginal contents were therefore conducted every four hours when the females were in a mating stage.

Experimental Groups

A total of 15 groups of animals have been included in the study. Three females were sacrificed at each of the following times: the 6th day of pregnancy, the 10th day, and at 1-day intervals thereafter to parturition. Three non-pregnant animals and three females and their newborn young were also sacrificed. If the number of young being produced

at any stage fell below nine, the animal was discarded and another female was assigned to the group.

The Ration

The stock diet used in this laboratory is a modification of the Steenbock stock ration (Steenbock, 1923). Dried whole milk has been incorporated into the mixture of grains, trace elements have been added, and in addition the animals received three supplements: cod liver oil, raw beef, and fresh carrots. The ration consisted of the following ingredients:

Yellow cornmeal	56
Linseed meal	16
Casein	5
Alfalfa meal	2
$CaCO_3$*	0.5
NaCl	0.5
Yeast	9.5
Yeast (Irradiated)	0.5
Wheat germ	10
Dried whole milk	33

Supplements:

5 grams raw beef)
10 grams carrots) Each fed 3 times per week

50 milligrams cod liver oil daily

*Trace elements added: KI, $MnSO_4$, $Al_2K_2(SO_4)_4$, $CuSO_4$

At the beginning of the study a surplus of all items of the diet was purchased so that the composition of the ration would remain the same throughout the entire investigation.

Perishable products were stored in the frozen state while grains, yeast, wheat germ, and dried whole milk were stored in a cool room.

Preparation of Tissues for Future Vitamin Assays

Since the concentration of vitamins in the liver is believed to denote the nutritive state of the animal the maternal hepatic tissue was prepared separately for subsequent vitamin analyses. Other body stores were considered as a whole and were estimated by analyses of the carcass of the mother. The carcass in all cases included the entire animal except for the hair and skin and the digestive tract and its contents. In the case of the pregnant animals, vitamin concentrations were determined for placental and fetal tissues at each interval of the gestation period. Earlier than the 13th day of pregnancy these tissues were too small for satisfactory division and the uterus and its contents were treated together.

Animals were killed by intrapleural injection of a lethal solution of sodium pentobarbitol (Nembutal). The uterus was removed and the young were examined carefully. Any evidence of resorptions was recorded. Photographs of the developing young were obtained at each stage of pregnancy. Newborn animals were weighed individually, but during prenatal

stages the entire litter was weighed. The fetuses were transferred to a Waring blendor, a small amount of chloroform was added, and following the death of the young the tissue was blended. The tissue was finally diluted to a suitable weight with distilled water and acetate buffer (pH 4.6-4.8).

The weight of the total placental tissue was recorded and the tissue was treated in a manner similar to that described for the fetuses. The maternal liver was excised, dipped into distilled water to remove free blood, patted free of surplus moisture with filter paper and carefully examined for color and signs of defects. After this organ was washed it was thoroughly mixed in the Waring blendor with acetate buffer and distilled water. Hepatic tissue was diluted to a final weight of approximately 250 grams.

The skin and hair of the female were removed and the digestive tract was detached. Carcass weight was calculated from the original weight of the animal minus the weight of the waste, the liver, the fetuses and the placentae. This tissue was first ground in a meat grinder to facilitate final mixing in a Waring blendor. Eventually the carcass was diluted to a weight of approximately 500 grams, and a portion of the mixed tissue was saved for future vitamin assays.

All tissues were stored in one-half pint canning jars at 20°F. until vitamin assays could be completed. Prior to the removal of aliquots for vitamin assays, the samples were

thawed at room temperature, and the tissues were reblended to assure uniformity.

RESULTS AND DISCUSSION

The rate of growth of the females, the number of young produced during first pregnancies, and the weight and generally good condition of these young at birth have led us to believe that the females selected for the present study were in excellent health and were able to reproduce normally. Apparently the stock ration provided ample amounts of all essential nutrients for rapid growth and for satisfactory reproduction.

The weaning weight of the females at 28 days of age averaged 49 grams. This value agreed well with observations of others (Spivey, 1947, and Bear, 1950) and seemed to indicate that the stock young assigned to this study were comparable to those produced during previous years. The animals were mature at approximately 42 days of age, the opening of the vaginal orifice being used as the criterion of sexual maturity. This figure also agreed closely with earlier observations of healthy stock females of this colony. Growth during the six-week period directly following weaning averaged 15.5 grams per week, again illustrating satisfactory performance of the animals.

Females mated promptly and produced an average of 8.5 young during first pregnancies. With few exceptions the

young were active at birth, normal in color, fairly uniform in size, and free from defects. The average birth weight of first litter young was 4.9 grams.

Animals sacrificed at the various intervals during second pregnancies likewise appeared in good condition. Autopsies revealed no evidence of infections or other abnormalities. Of 463 implantation sites observed for this group of females, 27 resorptions were found. This incidence of resorptions (5.8 per cent) is considerably lower than has been reported by other workers in this laboratory (Armstrong, 1937), although data on the frequency of resorptions in stock animals are limited.

There was good agreement between the weights of the placental and fetal tissues of the three females sacrificed at each interval during pregnancy. It will be observed from Figure 24 that placental tissue increased in weight gradually from an average of 3.6 grams on the 15th day of pregnancy to a maximum of 7.4 grams on the 21st day. Fetal tissue which weighed only 1.5 grams on the 13th day of the gestation period increased rapidly, particularly from the 18th day of pregnancy to term. The average weight of the newborn rats was 55 grams per litter.

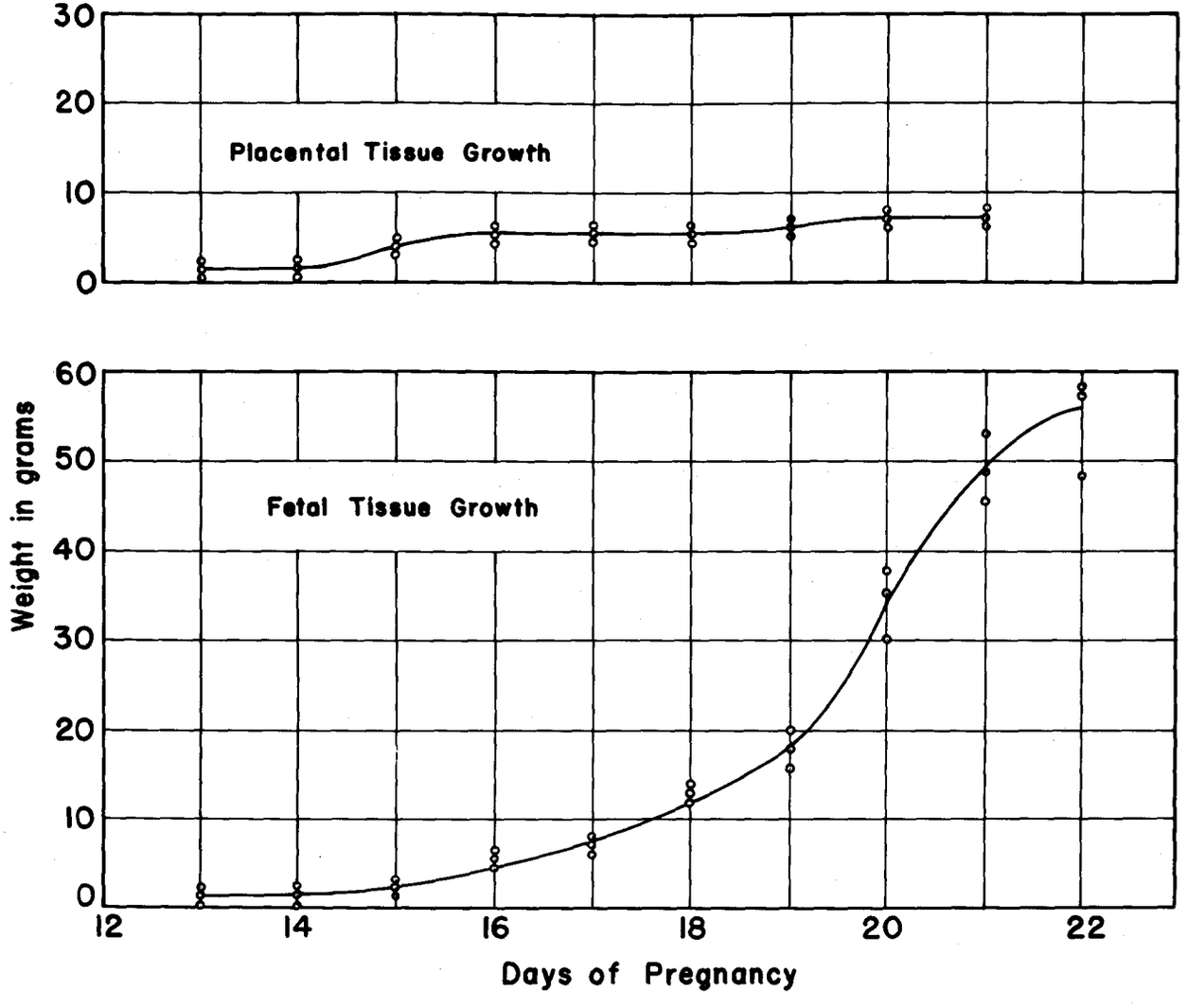

Fig. 24. Growth of tissues at various stages of pregnancy.

Photographs of the fetuses and the uterus at the various intervals during pregnancy will be presented on the following pages. These pictures (Figures 25 to 44, inclusive) portray the embryological development of the rat produced by stock animals of the colony of the Foods and Nutrition Department. Statements of the outstanding features of the fetus at each age are given. Satisfactory development of the young was again reflected from these records.

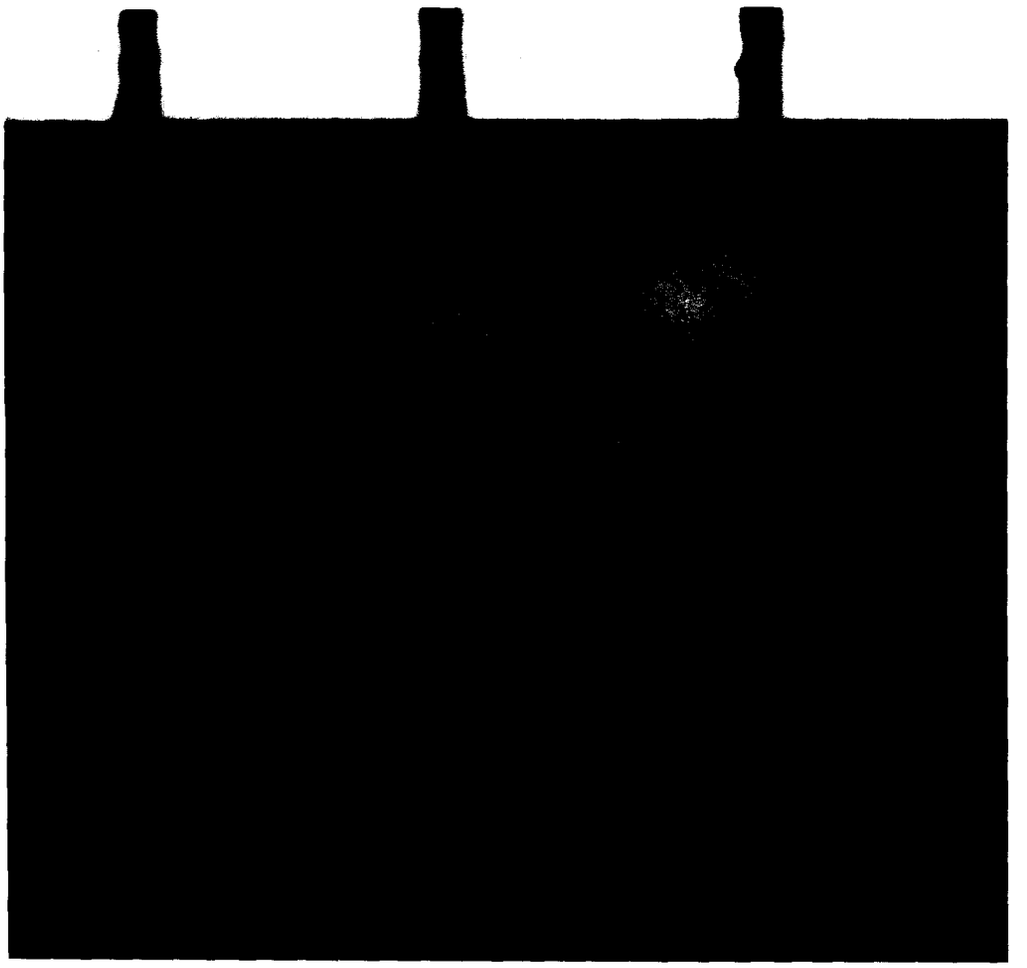

Fig. 25. Embryo at 10 days of pregnancy.

This embryo is approximately 2 millimeters in length. The primary regions of the brain are distinguishable. The body is dorsally convex, bending over the heart. The position of the future brachial arches is evident. The eye pit is distinct.

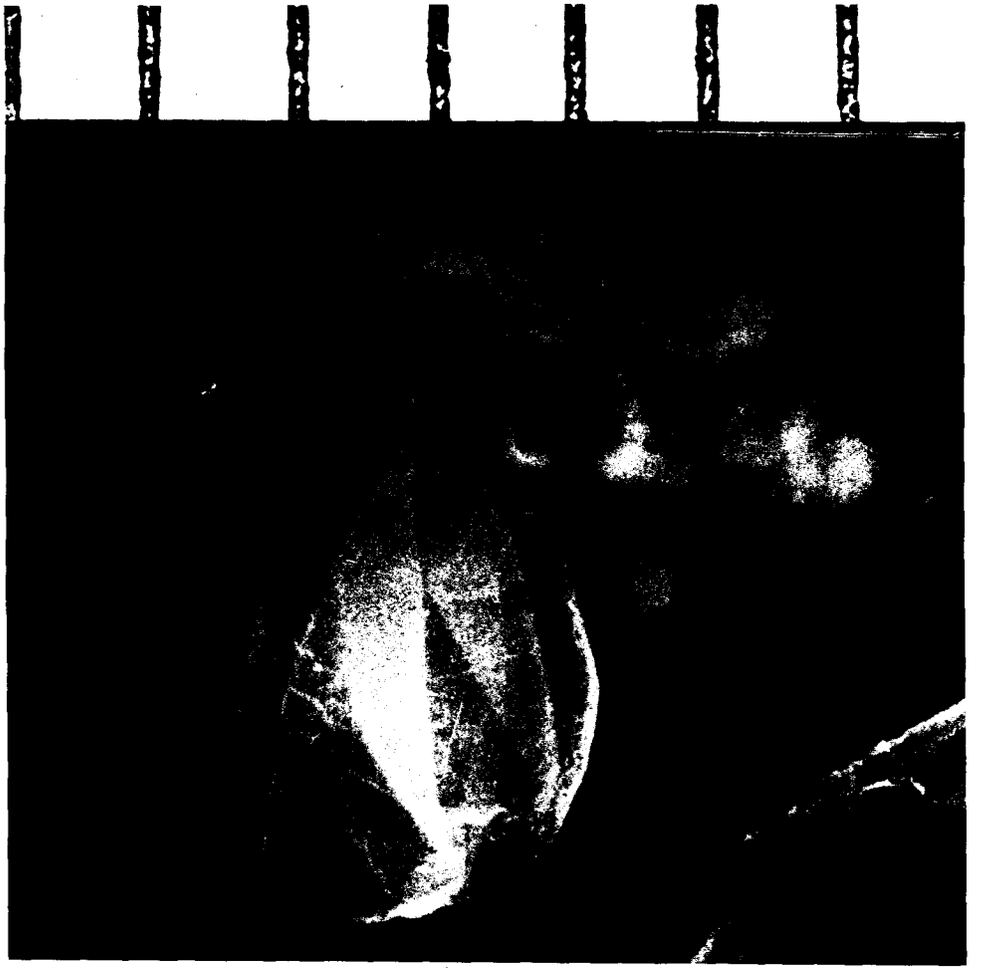

Fig. 26. Embryo at 11 days of pregnancy.

The embryo is 8 millimeters long. The somites are clearly visible along the posterior region. The eye pit is visible. The mandibular arches are widely separated. The embryo is concave at this age.

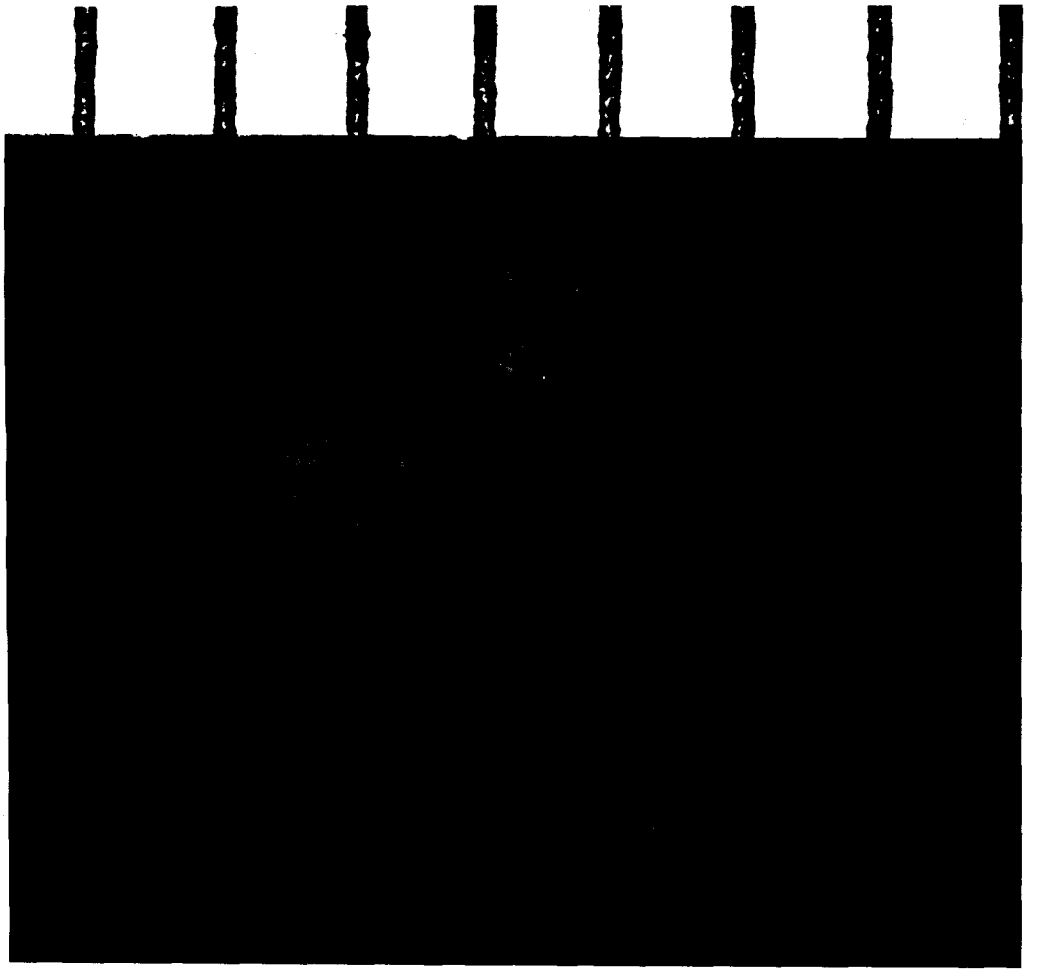

Fig. 27. Embryo at 12 days of pregnancy.

The embryo is 11.5 millimeters long. This photograph shows the divisions of the brain clearly. The mandibular arches are just anterior to the heart. The limb buds are very clear in this photograph. Posterior to the brachial arches, a small dark region locates the auditory pit. A portion of the amnion is seen in the lower left corner of the picture.

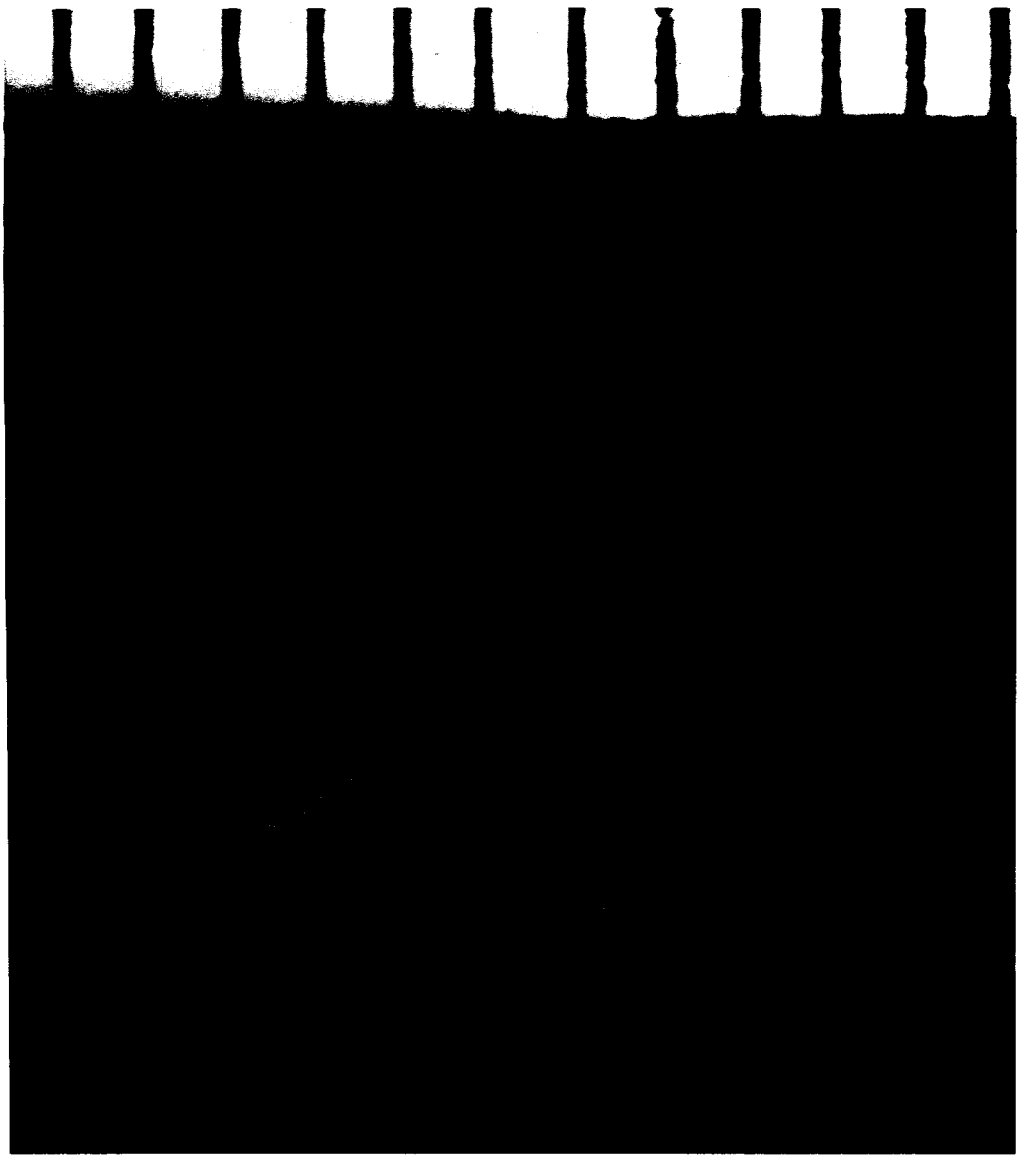

Fig. 28. Embryo at 13 days of pregnancy.

The embryo is 12 millimeters long. In this photograph there are minor advancements from the previous day. The two cerebral hemispheres are apparent in this animal.

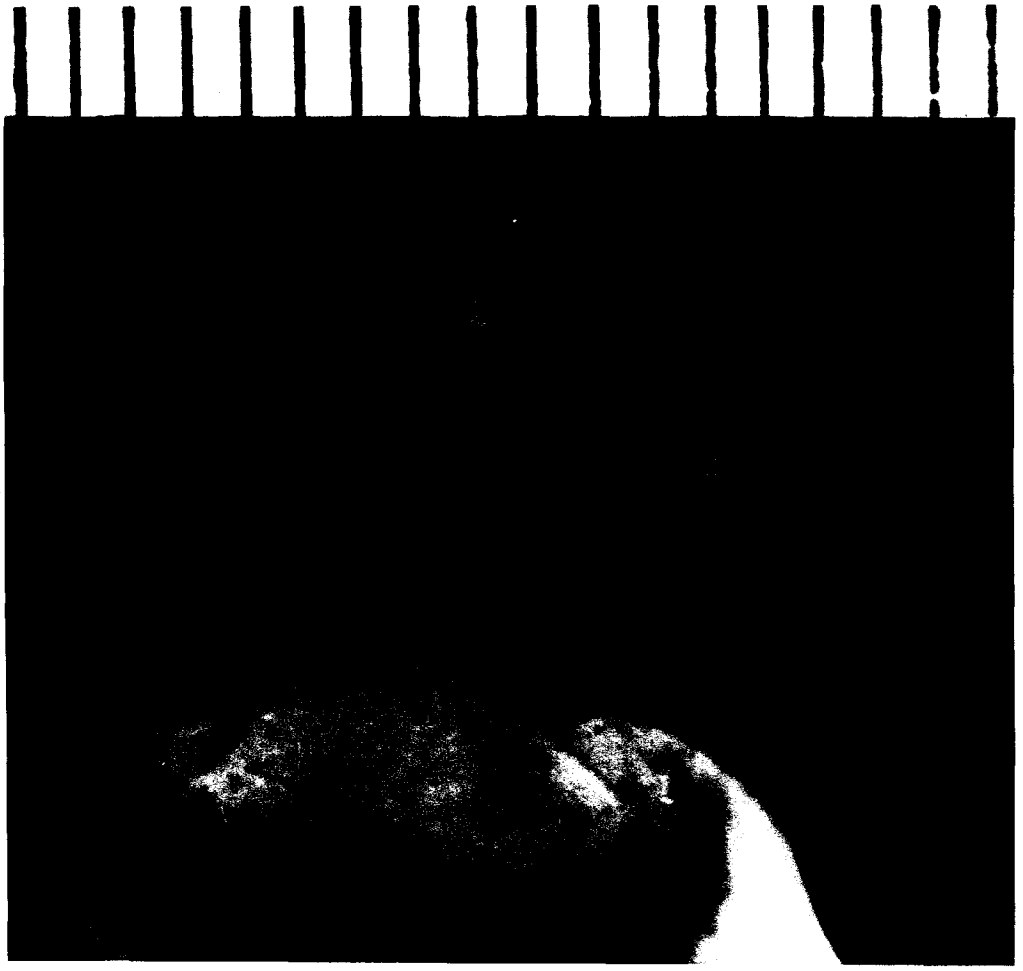

Fig. 29. The fetus at 14 days of pregnancy.

The fetus is approximately 12.5 millimeters in length. The body curvature has lessened. The head is well developed and is very large in proportion to the rest of the body. The nasal and maxillary processes have fused to form the upper jaw. The eye is open; the ear is clearly visible. The digits in the anterior and posterior limbs are visible.

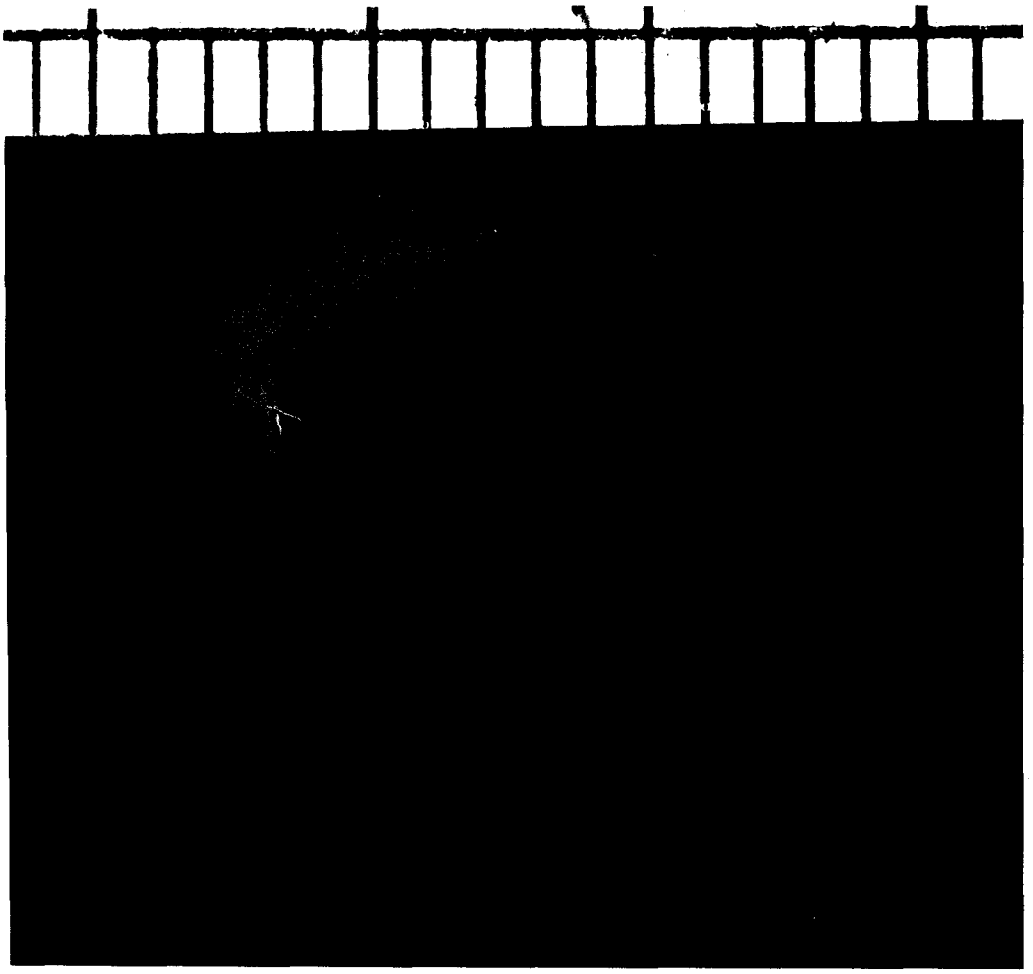

Fig. 30. The fetus at 15 days of pregnancy.

The fetus is approximately 13 millimeters long. The anterior and posterior extremities have developed joints. The feet are angular and webbed. The face region of the fetus is well developed. The head is still large in proportion to the rest of the body. There is no neck. The mesencephalon of the brain is very pronounced. The skin has become thicker, making the blood supply of the animal less obvious.

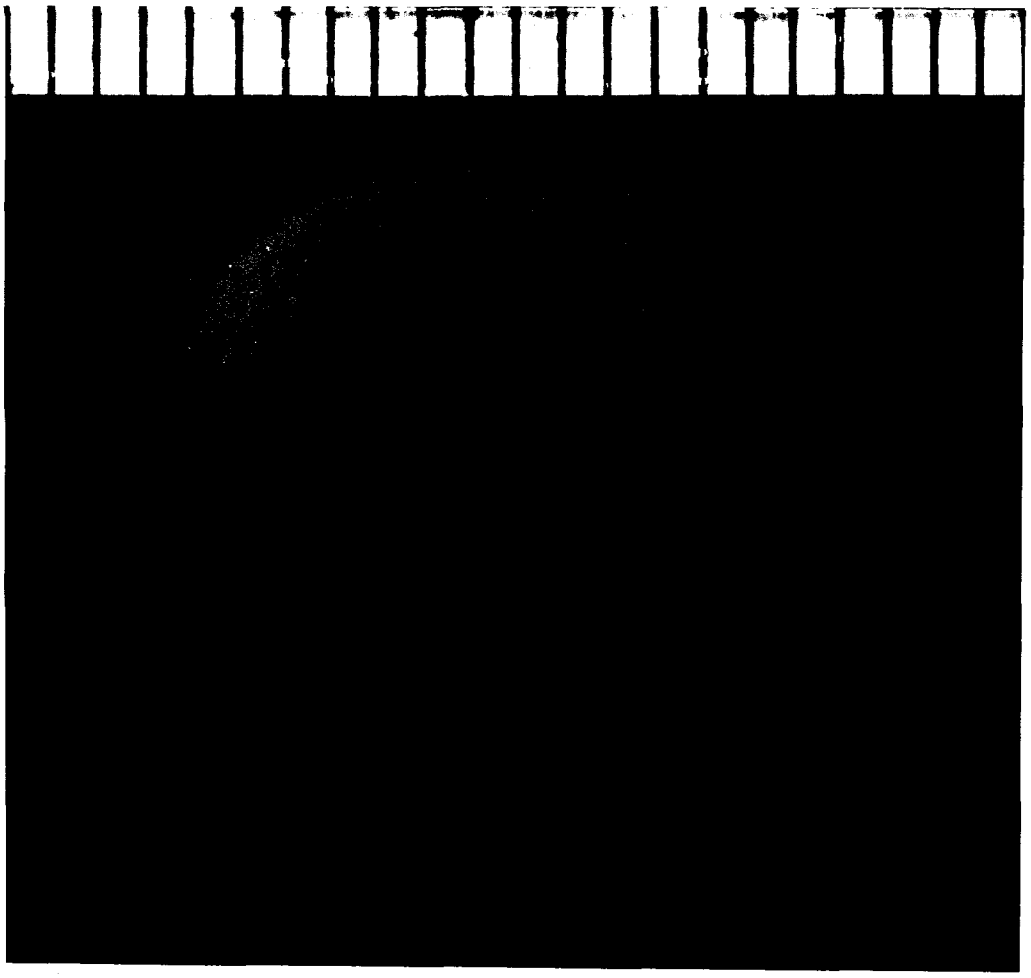

Fig. 31. The fetus at 16 days of pregnancy.

The fetus is approximately 14 millimeters in length. There seems to be very little advancement beyond that observed in the former photograph. The anterior extremities are more advanced than the posterior; both are still webbed. The forehead protrudes and is curved.

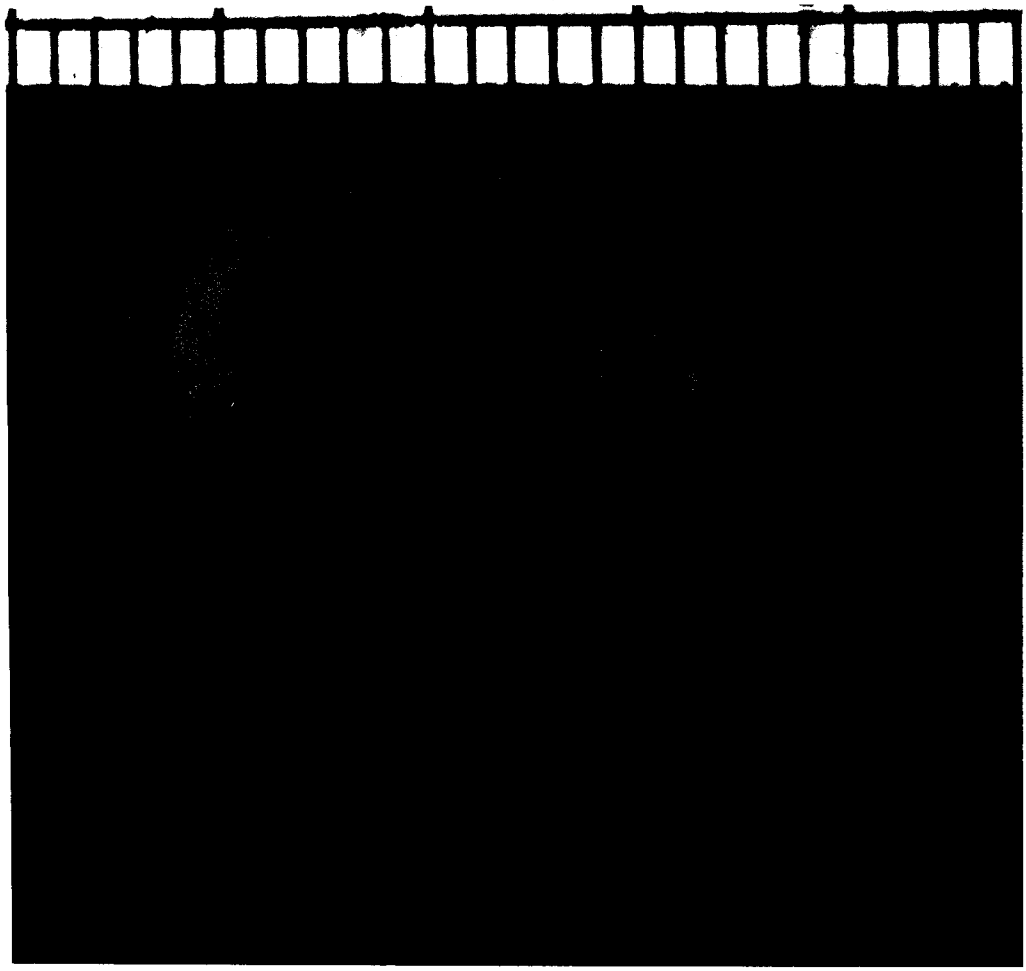

Fig. 32. The fetus at 17 days of pregnancy.

The fetus is approximately 16 millimeters long. The head size in proportion to the rest of the body is smaller at this age. The heart region has reduced in size and the abdominal region has increased. The forehead does not protrude as much as formerly. The mesencephalon is less pronounced. The eyes and ears are both open. Vibrissae are seen on the snout.

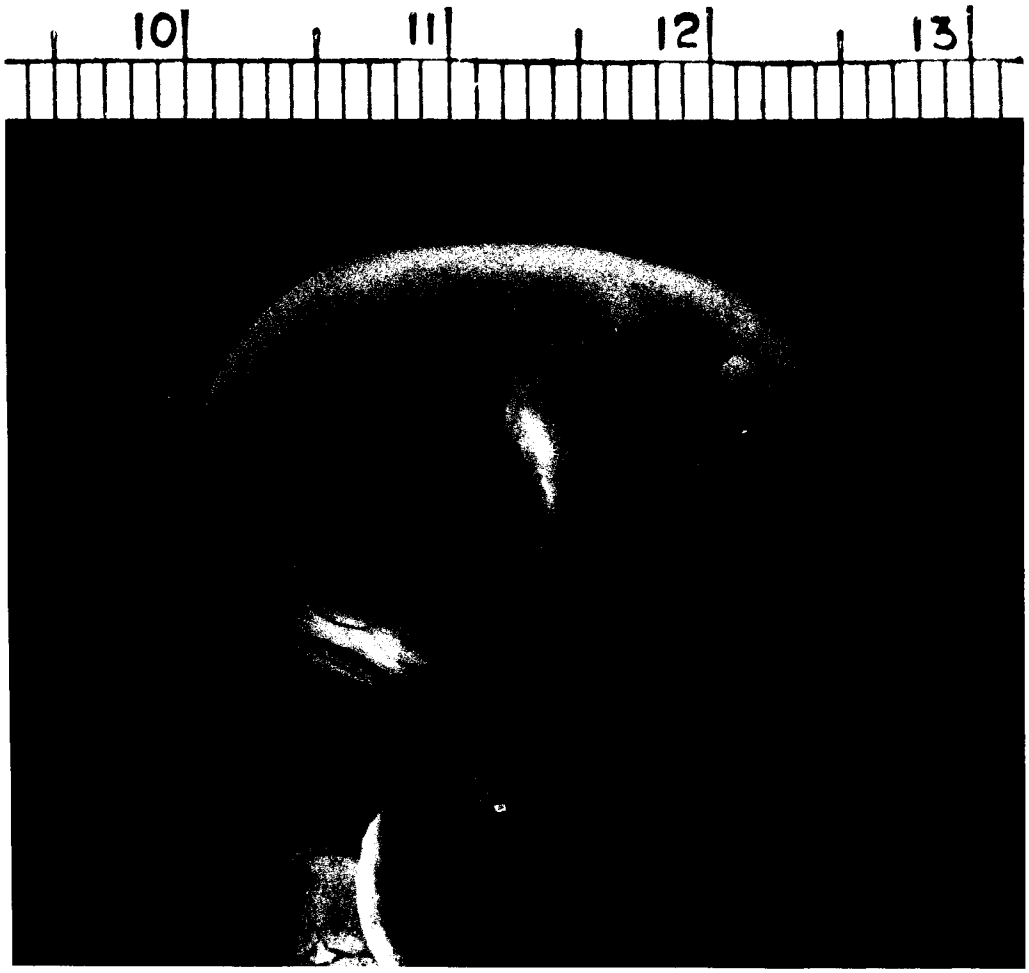

Fig. 33. The fetus at 18 days of pregnancy.

The fetus is about 22 millimeters in length. This fetus is less curved than younger ones. The head is well rounded; the protuberance of the mesencephalon has disappeared. The posterior limbs are well developed; however, the clefts between the digits of the feet have deepened. The vibrissae are distinct. The eyes and ears are closed.

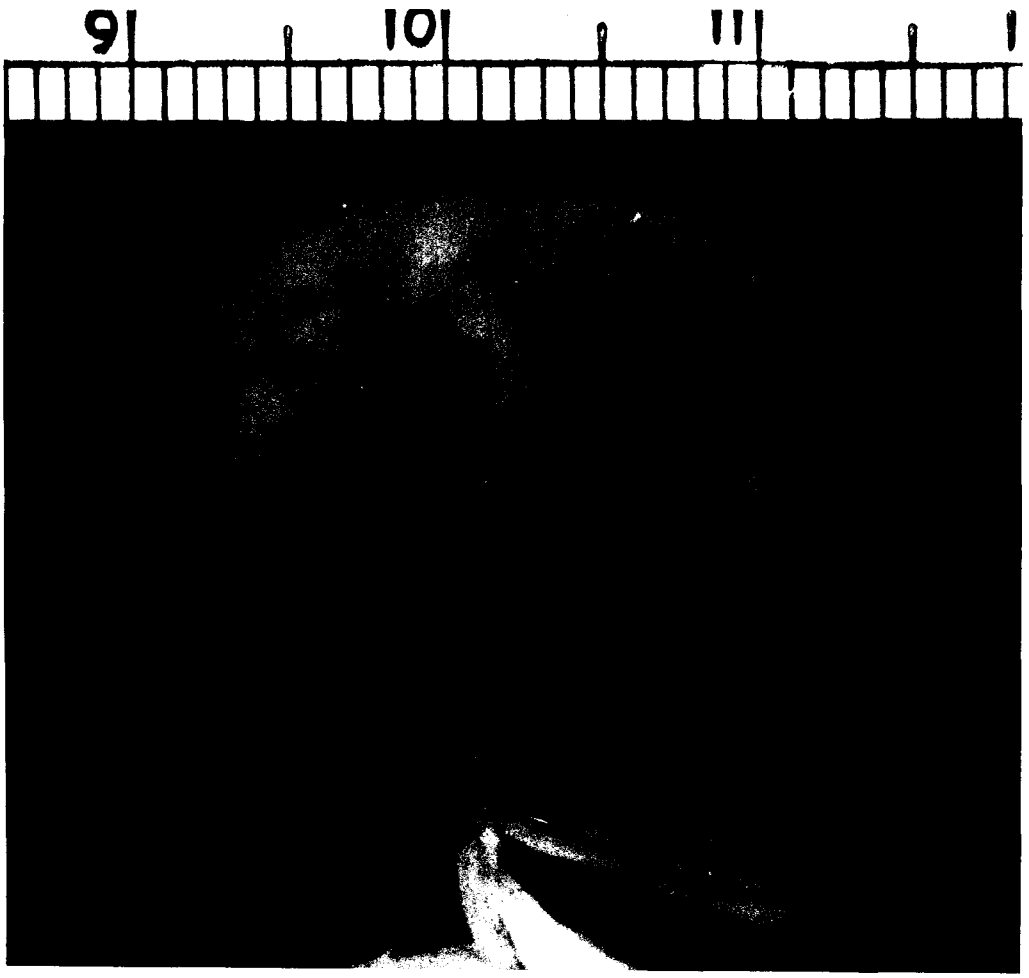

Fig. 34. The fetus at 19 days of pregnancy.

The fetus is 25 millimeters long. This figure shows the fetus covered with vibrissae. The external ear is prominent. The posterior limbs resemble those of the adult rat. The head has decreased in size in comparison with the rest of the animal. The neck is well formed at this age.

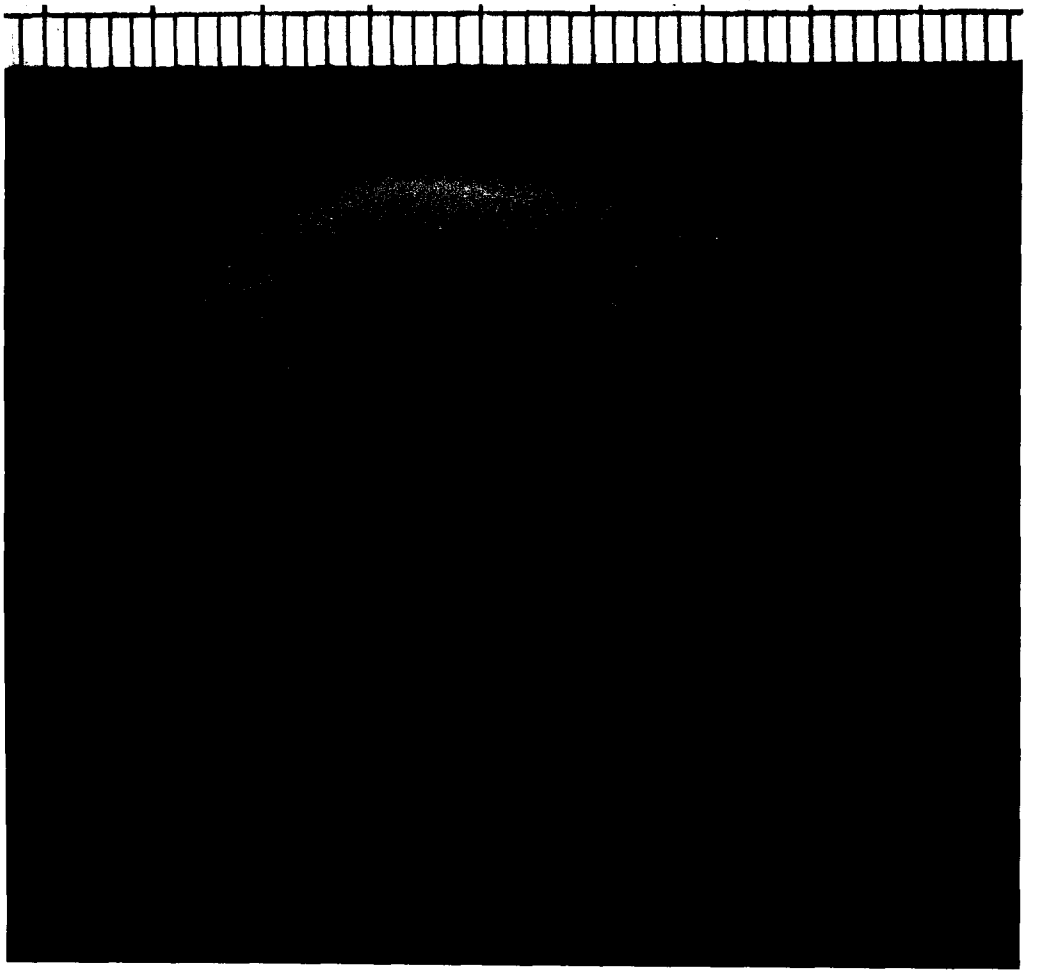

Fig. 35. The fetus at 20 days of pregnancy.

The fetus is approximately 32 millimeters long. The one in this photograph is beginning to develop the wrinkled skin of the newborn. The eyes and ears are closed; the mouth is open. The general appearance of the fetus is more like that of the young at term.

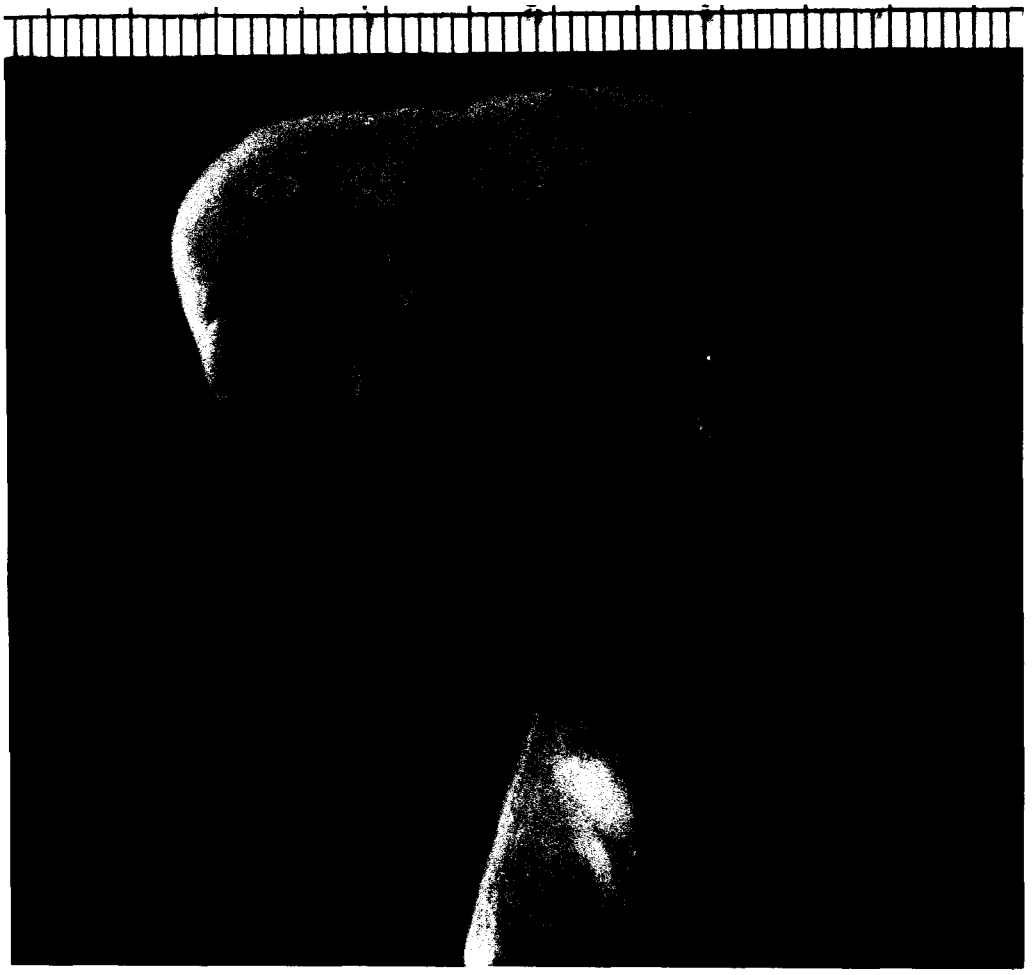

Fig. 36. The fetus at 21 days of pregnancy.

The fetus is approximately 41 millimeters long. At this age the fetus resembles the newborn rat. The placental attachments are clearly seen in this photograph.

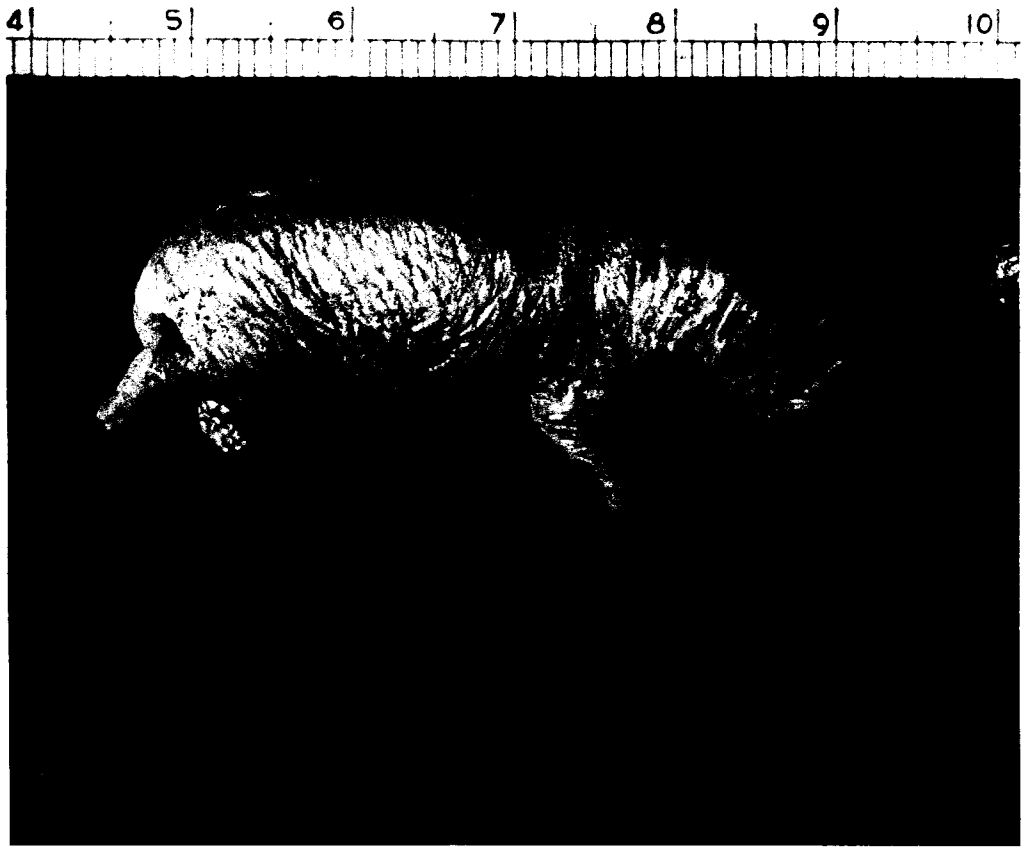

Fig. 37. The newborn rat.

At birth the rat has no hair, its skin is very wrinkled, and its eyes are closed. Its weight is approximately 5 grams. Its length is approximately 50 millimeters.

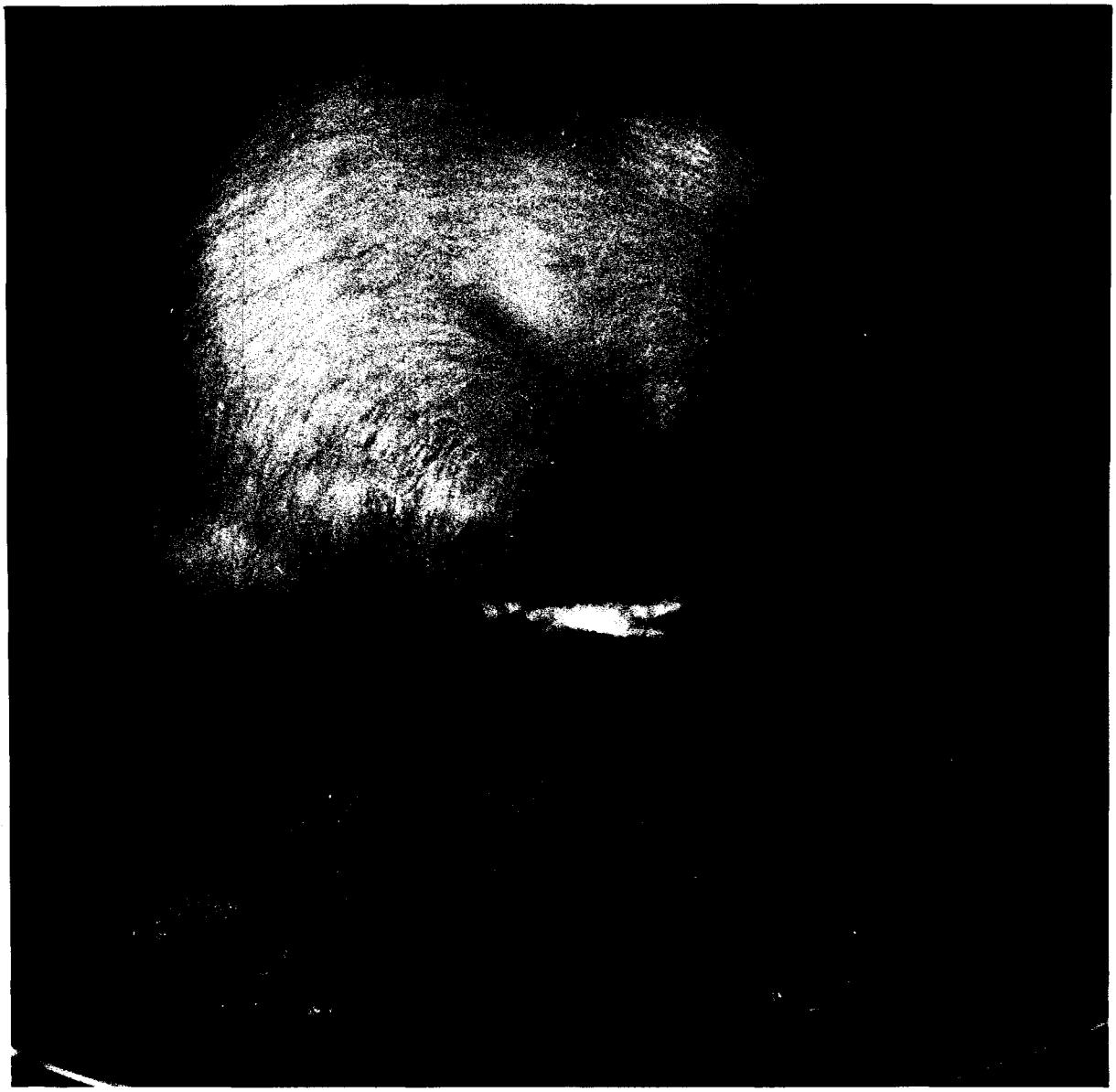

Fig. 38. Experimental animal number 47751.

This photograph shows a typical litter of newborn young.

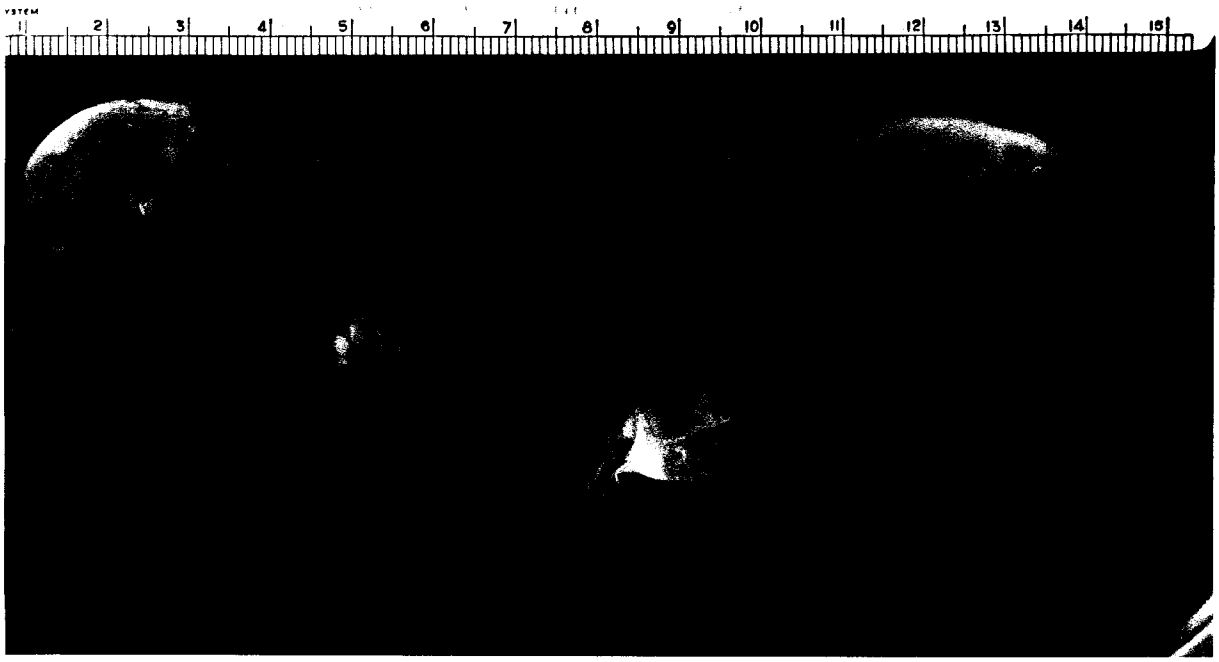

Fig. 39. Placental attachments and fetal membranes.

This photograph shows the membranes covering the fetus. The second animal is covered by the amnion. The umbilical cord, amnion and discoidal placenta are shown in the center figure. At the extreme right the discoidal placenta is pictured separately.

Fig. 40. Uterus of the non-pregnant rat.

Note the two horns and their attachments to the vagina. This uterus is 7.3 millimeters in length and weighs 1.2 grams.

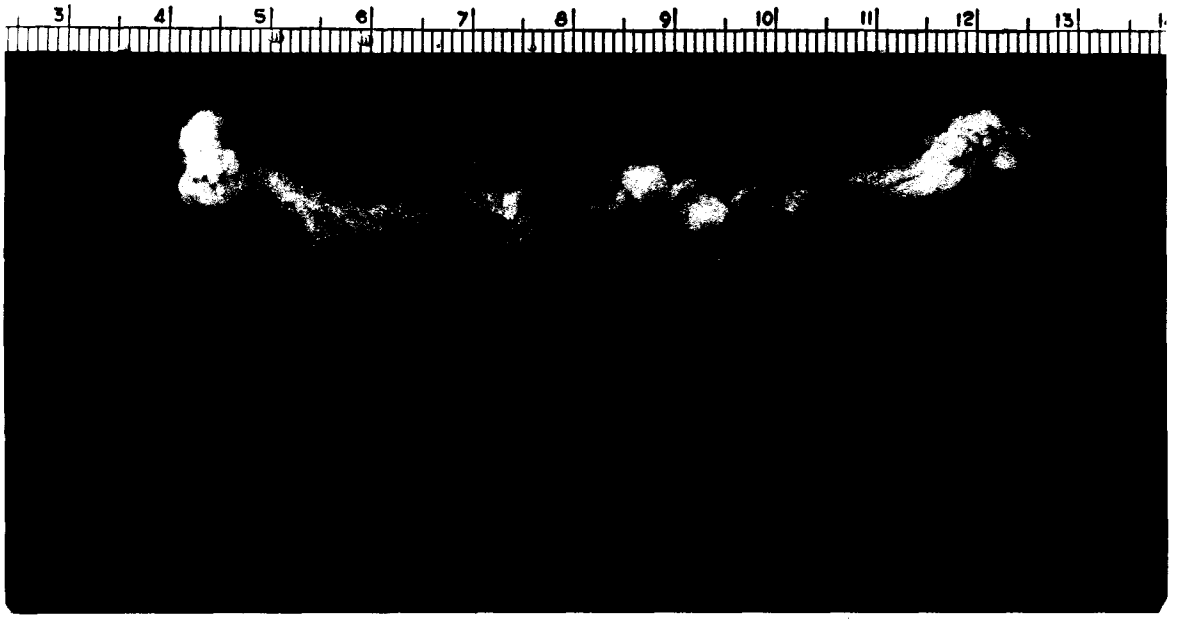

Fig. 41. Uterus on the sixth day of gestation.

This uterus is 8.3 millimeters in length and weighs 1.6 grams. The enlargements along the uterine horns show the location of the implantation sites. These sites are more distinct on the antimesometrial than on the mesometrial border.

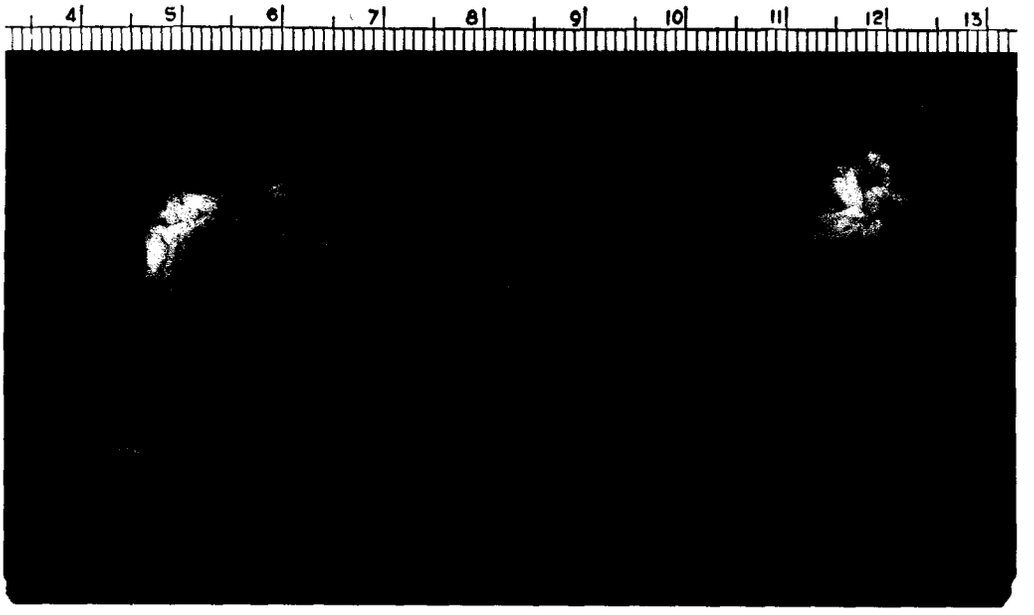

Fig. 42. Uterus on the 10th day of gestation.

The implantation sites are easily seen on the mesometrial as well as on the antimesometrial border. This period is during the transition stage when the embryo is attached to both borders. This uterus and its contents weighs 2.4 grams.

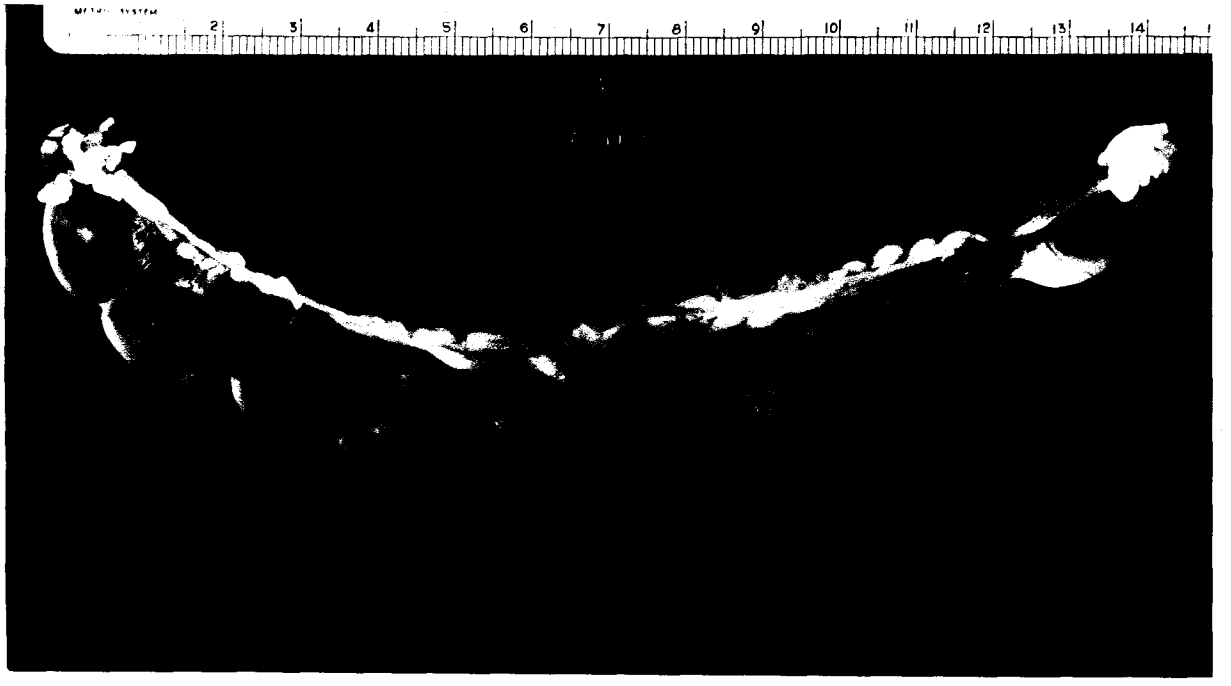

Fig. 43. Uterus on the 17th day of gestation.

The fetuses in this photograph are attached to the mesometrial portion of the uterus. Resorptions have occurred at the second and fifth implantation sites from the right of the figure. This uterus and its contents weigh 20 grams.

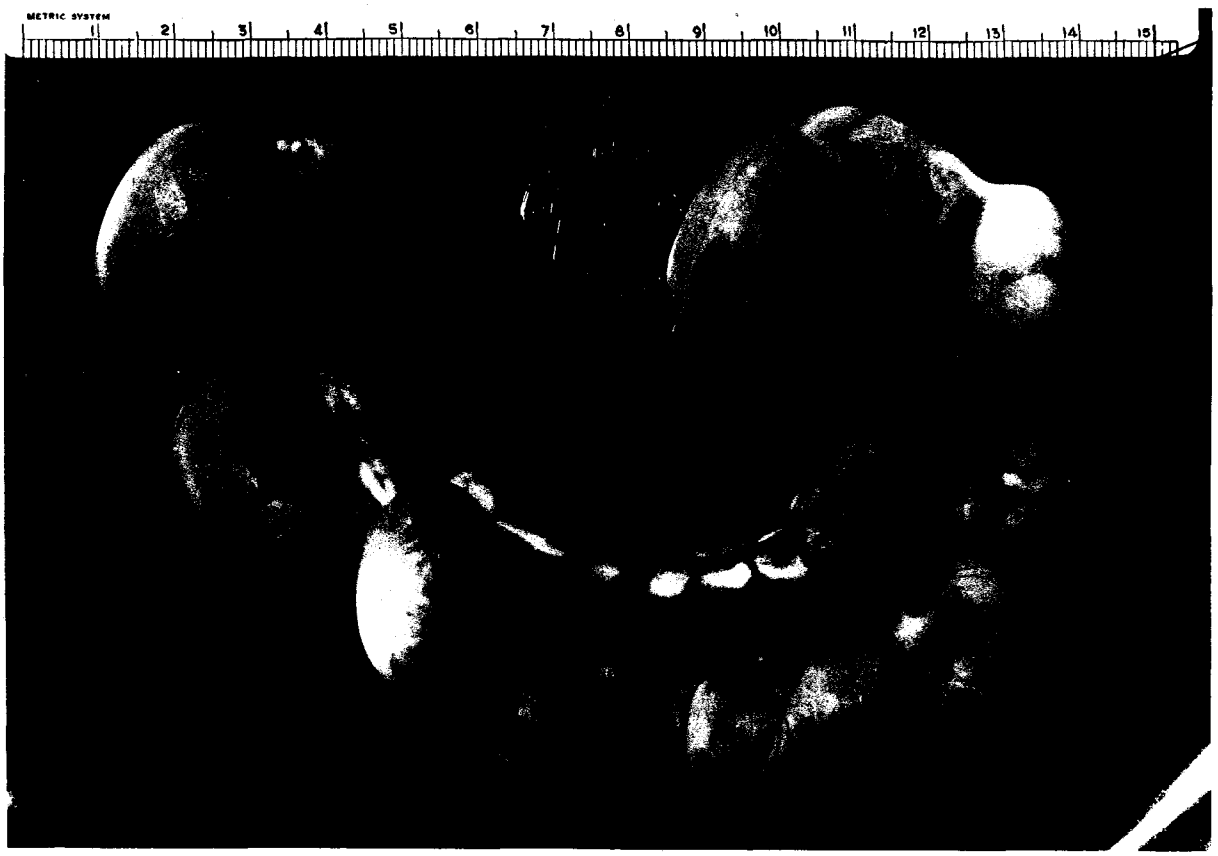

Fig. 44. Uterus on the 21st day of gestation.

On the 21st day of pregnancy one can clearly see the entire shape of the individual fetus through the uterine membrane.

SUMMARY AND CONCLUSIONS

Lack of quantitative information concerning the increased needs of the B-vitamins during pregnancy in rats and particularly their needs at different stages of the gestation period has stimulated the writer to pursue a new approach to estimating vitamin needs during reproduction--that of measuring the rate of deposition of the vitamins in the developing fetal and placental tissues.

A series of healthy stock rats have been sacrificed at intervals during their second pregnancies in order that the concentration of vitamins might be determined for the embryonic tissues of different ages. Maternal hepatic and carcass tissues were also prepared for vitamin assays to provide a means of determining whether vitamins stores of the mother changed as pregnancy progressed.

The growth of the experimental animals, their age at maturity, and their performance during first pregnancies indicated that the females selected for this study were equal to our customary stock rats which have reproduced satisfactorily for many generations.

Photographs of the developing fetus at twelve different ages are included in the report.

A detailed account of the embryological development of the rat is presented.

PART II

THIAMINE REQUIREMENTS DURING REPRODUCTION

Information concerning the extent to which reproduction increases the thiamine needs of the mother is extremely meager. Surprisingly few records are available on the quantity of thiamine consumed by women during pregnancy which have been correlated with the absence or presence of clinical signs of thiamine deficiency in the mother or the infant. The extensive work of Baker and Wright (1936) in areas where beriberi was endemic has shown without question that thiamine intakes of as little as 200 to 500 micrograms per day are inadequate. Polyneuritis of pregnancy was prevalent among women in these sections.

Recently Oldhan and coworkers (1947) have published data concerning the thiamine requirements of women during pregnancy which indicate that the need is raised only slightly at this time. The Chicago workers found that pregnant women who ingested one milligram of thiamine daily excreted approximately 200 micrograms of the vitamin in the urine. According to our present interpretation of the excretion of vitamins by the kidneys, their data indicated that the women were amply supplied with thiamine. Prior to the study of Oldham and her associates, it was generally thought that much larger quantities of thiamine were advisable during pregnancy.

This concept has resulted from many studies, among which are those reported by Toverud (1940) and Lockhart et al. (1943). Toverud observed that when pregnant women were given 1 to 3 milligrams of thiamine daily for as long as 17 days, little of the vitamin was excreted in the urine. If the amount was raised to 4 or 5 milligrams per day the quantity of B_1 excreted by the kidney was equal to that observed for well fed non-pregnant women. Lockhart gave thiamine orally and intermuscularly to both pregnant and non-pregnant women and found that it took approximately three times as much of the vitamin for pregnant women to reach the "excretion peak" (the point at which the highest proportion of administered thiamine was excreted) as was required for non-pregnant women. Unfortunately in the two studies just described the authors had no record of the previous thiamine intakes of the pregnant women. In the light of the work of Oldham et al., it might be assumed that the subjects of Toverud and Lockhart had been rather severely depleted in B_1.

The quantity of thiamine recommended during pregnancy is 1.5 milligrams per day (National Research Council, 1948). This amount is 300 micrograms higher than the value proposed for non-pregnant women who consume the same number of calories, i.e., 2400. According to the work of Oldham and her associates, the allowance suggested during pregnancy provides a 50-per cent margin of safety. This recommendation would

seem wise to insure against shortages due to disturbances such as nausea and lack of appetite. Also this margin of safety allows for minor reductions in total food intake without endangering the thiamine supply. Some curtailment in total calories is frequently recommended by physicians if the mother has gained considerable weight.

One might conclude from our present information that the thiamine needs of women are not significantly increased during pregnancy unless unusual complications occur. This conclusion means that a wisely chosen diet will furnish ample quantities of thiamine and that in most cases supplements of the pure vitamin or concentrates are not beneficial.

A search of the literature for definite data concerning the quantity of thiamine needed by the rat for reproduction revealed that few workers had explored this question. Several groups of investigators who were interested in formulating synthetic rations satisfactory for reproduction in rats had selected thiamine supplements which varied from 20 to 200 micrograms per day. In the majority of these studies the rations proved to be incomplete and reproduction was unsatisfactory. Until recently biotin, folic acid, and B_{12} were not provided in adequate amounts so that the outcome of pregnancy was influenced by such omissions as well as possibly by inadequate amounts of thiamine at the lower intakes. These studies have thrown little light on the quantitative needs

for thiamine during reproduction.

The satisfactory performance of stock animals of the colony of the Foods and Nutrition Department denoted that these animals received adequate intakes of thiamine during reproduction. Records of the customary food intakes of females during gestation and the concentration of thiamine of the ration revealed that the animals had access to approximately 150 micrograms of thiamine per day. Just how much surplus vitamin this diet provides and what demands for thiamine are imposed by pregnancy are questions which have been undertaken for investigation in the present study.

The absence of satisfactory information concerning the quantitative needs for thiamine during pregnancy in the albino rat has encouraged the writer to investigate two procedures in an attempt to contribute information on this topic. One approach has been to measure the deposition of thiamine in normally developing fetuses and to observe the thiamine stores of the pregnant animals at various periods during gestation. The second procedure has been to determine the intake and urinary excretion of thiamine of both pregnant and non-pregnant littermates which were fed the stock ration.

EXPERIMENTAL PROCEDURE

Detailed information regarding the selection and care of the animals, the ration, the mating procedure and the preparation of the tissues for vitamin analyses has been given in Part I (pages 49 to 55).

Thiamine Assay Procedure

All tissues were allowed to thaw at room temperature and were then reblended in a Waring blendor before aliquots were removed for analyses. The quantity of tissue sampled in most cases contained approximately 5 micrograms of thiamine. This preparation was suspended in acetate buffer (pH 4.4-4.6) and 200-milligram quantities of both papain and takadiastase were added. The samples were covered with a thin layer of toluene and were then incubated for 18 to 24 hours at 37°C. Following the enzymatic treatment the samples were filtered and diluted to a known volume. Two aliquots of each preparation of tissue, containing approximately one microgram of thiamine, were passed over activated decalso. The vitamin was eluted with acidified potassium chloride and the samples were finally brought to a volume of 25 milliliters. Thiamine was determined as thiochrome according to the procedure

recommended by Hennessy (1942). Readings were made in a Coleman photofluorometer.

In certain cases the quantity of tissue available was extremely limited so that the amount of thiamine in the assay was necessarily reduced.

The thiamine content of the ration was determined in the same manner as that described for the tissues. Urine specimens collected during the metabolism studies were assayed similarly except that the enzymatic treatment was omitted.

The Metabolism Studies

Ten stock females approximately 42 days of age were transferred to individual metabolism cages and urine was collected quantitatively for the following 24 days. The metabolism periods were 4 days in length. At the close of the sixth collection period the females were put in their customary cages and were mated with stock males as quickly as possible. When pregnancy was established, as evidenced by the presence of sperm or the copulation plug the females were returned to metabolism cages. At this time a non-pregnant littermate was also started on the seventh metabolism study. Five females were mated and five animals were studied as non-pregnant controls. The five females mated promptly so that the metabolism studies were not interrupted longer than 4 days. During the

early portion of pregnancy the collection periods were 4 days long. However, the time was shortened to 2 days near the end of pregnancy to make it possible to detect more narrow changes in excretion of certain other vitamins which were being studied at the same time.

The metabolism cage used in the present work consisted of a circular cage made of a 1/2-inch wire mesh with a bottom raised 2-1/2 inches. The cage was placed on a pyrex pie plate 10-1/2 inches in diameter and was held in position by 3 or 4 S-shaped metal holders which gripped the edge of the glass plate and the cage. A circle of fine wire screening, cut slightly smaller than the circumference of the cage, was suspended approximately one inch above the base of the pyrex plate, due to the tapered shape of the dish. This screen allowed the feces to collect above the plate and lessened the problem of contamination of the urine. Fine hair of the animals also collected on this screening. The feces were removed with tweezers every 24 hours and were discarded.

At the beginning of each collection period the animals were weighed and were put into a clean metabolism cage which contained a weighed amount of stock ration. The animals ate ad libitum, and records were kept of their food consumption. The food cups consisted of cosmetic jars approximately 2 inches high and 3 inches in diameter. The cups were wired

securely to the cage to avoid spillage. Tight-fitting metal lids for these containers were prepared to prevent scattering of the ration or the possibility that the rat might rest in the food cup. A circular hole approximately one inch in diameter was drilled in the covers. Water was supplied from the outside of the cage by means of a crystal fountain.

Since the stock diet customarily used in this laboratory included supplements of both raw beef and carrots, it was found most satisfactory to feed carrots during the first 2 days of each collection period so that if pieces of the vegetable fell between the wires of the cage the supplement could be recovered and placed in the food jars. The meat supplements were consumed eagerly and introduced no problem.

At the termination of each collection period the animal was transferred to a new cage. Fecal samples were removed from the wire screening and fine hairs were removed by means of a camel's hair brush. The pyrex plate, the fine wire screening, and the floor of the cage were washed carefully with a fine spray of distilled water. A rubber policeman was rubbed over these surfaces to facilitate the removal of the urine. The washings were transferred to a volumetric flask through a funnel containing loosely fitted cotton which served to remove extraneous matter. After several rinsings of all surfaces and the cotton the urine was diluted to a volume of 250 ml. A portion of the well mixed sample, which

represented the urine for four days, was covered with toluene and stored in a refrigerator until vitamin analyses could be completed. Since it was found that the urine contained considerable amounts of the vitamins, it was possible to dilute the samples to 250 ml., which provided ample liquid for satisfactory washing of the cages.

RESULTS AND DISCUSSION

Deposition of Thiamine in Tissues as a Means of Determining Thiamine Requirements for Reproduction

Data on the occurrence of thiamine in fetal and maternal tissues at various stages of pregnancy are presented in Table I and Figure 45. These results have proved interesting and have led the writer to believe that this procedure of studying vitamin requirements for reproduction in rats is a valuable one. It appears that the rat increases its hepatic stores of thiamine during pregnancy provided the ration is adequate. The increased concentration of B_1 is most marked during that portion of pregnancy when fetal growth is rapid, between the 18th and final days of gestation for the rat. During the first few days of pregnancy it was observed that the liver contained between 10 and 11 micrograms of thiamine per gram of fresh tissue. Toward the end of pregnancy the concentration had increased to 13 or 14 micrograms. No significant change in liver weight was encountered during the gestation period. The accumulation of thiamine in the liver may be advantageous for the female as she approaches that period of pregnancy when fetal growth is especially rapid.

Table I

Concentration of Thiamine in Fetal and Maternal Tissues

Period of pregnancy (days)	Rat number	Thiamine in tissue: Carcass Per gm. mcg.	Carcass Total mcg.	Liver Per gm. mcg.	Liver Total mcg.	Placentae Per gm. mcg.	Placentae Total mcg.	Fetuses No.	Fetuses Per gm. mcg.	Fetuses Total mcg.
Full term	47718	1.9	272	14.0	125	-	-	10	3.0	146.8
	47721	1.8	254	14.5	142	-	-	12	3.0	175.4
	47751	2.0	292	13.8	137	-	-	11	3.0	172.8
21	47717	1.9	284	14.9	130	3.2	20.9	10	3.4	158.6
	47749	1.8	271	13.3	124	3.1	23.5	11	3.2	155.7
	47799	1.9	248	13.3	130	4.1	34.9	10	3.0	160.4
20	47750	2.1	341	13.4	138	4.0	25.7	10	2.2	78.1
	47722	1.9	300	15.9	151	3.2	25.2	13	2.6	96.3
	48640	2.0	291	13.7	148	3.0	18.9	11	2.7	80.2
19	47798	2.0	305	13.5	131	2.5	19.0	11	1.7	30.8
	47902	1.8	267	14.0	132	3.1	18.3	9	1.9	39.7
	47895	2.2	326	13.0	121	2.6	14.9	9	2.3	39.5
18	47827	2.2	301	15.1	130	2.7	15.2	10	2.0	28.8
	47851	2.0	300	11.7	118	2.7	14.8	10	1.7	24.2
	47903	1.9	279	15.2	131	3.5	16.7	10	2.1	25.5

(Continued)

Table I (Continued)

Period of pregnancy (days)	Rat number	Thiamine in tissue: Carcass Per gm. mcg.	Carcass Total mcg.	Liver Per gm. mcg.	Liver Total mcg.	Placentae Per gm. mcg.	Placentae Total mcg.	Fetuses No.	Fetuses Per gm. mcg.	Fetuses Total mcg.
17	47912	2.2	318	13.3	118	2.9	13.1	10	1.8	11.6
	47854	2.0	296	13.4	121	3.0	16.1	11	1.8	16.3
	48472	2.0	310	11.0	115	2.2	9.2	9	1.6	9.8
16	47796	2.1	315	10.7	107	2.0	11.1	10	1.1	6.0
	47881	2.0	311	12.8	125	2.4	10.6	12	1.7	9.2
	48505	1.8	281	11.9	142	2.5	13.8	12	1.2	8.0
15	47927	1.7	240	10.9	108	2.3	8.1	11	1.9	5.1
	47913	2.1	292	11.0	106	2.5	8.6	11	1.8	4.3
	48513	1.9	254	10.0	100	2.2	8.1	12	1.9	6.4
14	47936	2.0	301	10.9	109	3.2	5.7	9	2.3	3.7
	47928	2.0	293	10.9	93	3.6	5.7	12	1.4	2.0
	47909	1.9	278	12.0	118	3.6	8.7	9	1.8	5.2
13	47828	2.3	320	10.5	101	2.3	4.8	10	1.0	1.2
	47896	2.3	338	13.8	108	2.2	5.6	13	1.4	2.2
	47777	2.1	357	12.1	115	2.5	4.0	12	2.0	2.2

(Continued)

Table I (Continued)

Period of pregnancy (days)	Rat number	Thiamine in tissue: Carcass Per gm. mcg.	Carcass Total mcg.	Liver Per gm. mcg.	Liver Total mcg.	Uterus and its contents* No. in litter	Uterus and its contents* Per gm. mcg.	Uterus and its contents* Total mcg.
12	47911	2.2	316	11.3	120	13	2.4	11.7
	47935	1.9	274	10.1	94	14	2.1	10.9
	47853	2.1	341	12.3	119	14	1.7	8.3
11	47925	2.2	305	10.5	105	11	1.7	4.9
	47882	2.1	329	11.8	118	15	2.3	8.6
	47937	2.3	341	11.0	102	13	1.9	5.3
10	48463	2.2	292	10.2	87	12	2.3	6.1
	48464	2.3	327	10.7	106	12	2.0	5.0
	48644	1.9	268	12.1	109	12	1.4	3.4
6	47852	2.3	306	11.0	88	-	3.4	6.1
	47778	2.3	321	9.3	77	-	3.2	4.1
	48646	2.2	286	12.1	109	-	3.2	5.1
Non-pregnant	47715	-	-	-	-	-	3.1	3.7
	47716	-	-	-	-	-	3.7	4.5
	47910	-	-	-	-	-	2.4	2.6

*Note difference in process prior to 13th day.

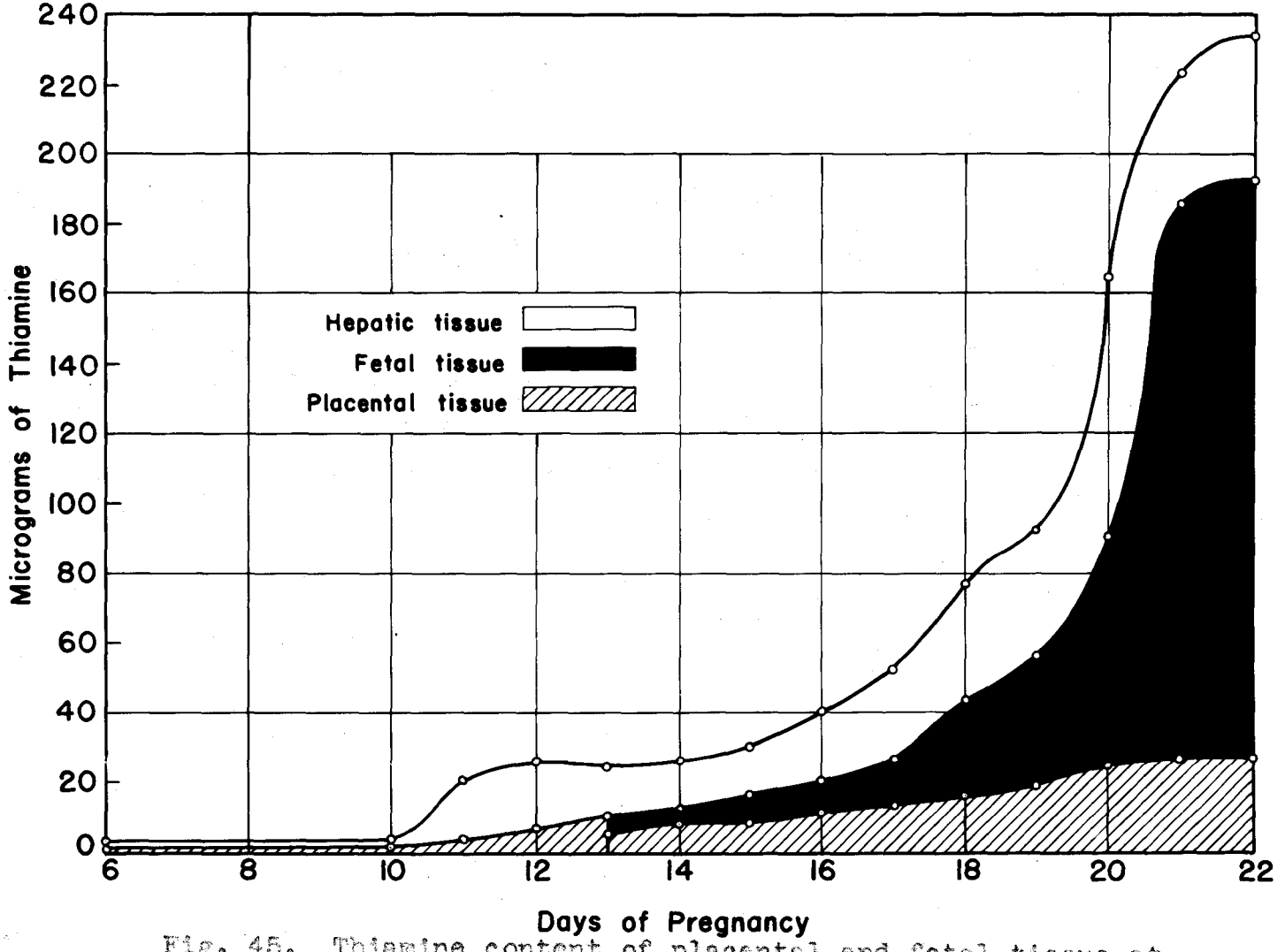

Fig. 45. Thiamine content of placental and fetal tissue at various stages of pregnancy plus increased thiamine observed in hepatic tissue during pregnancy.

This increase in hepatic stores of thiamine of the pregnant rat has not been mentioned before to the knowledge of the writer.

It will be noted from Figures 45 and 46 that the retention of B_1 to allow for the increased liver stores influenced the total thiamine requirement to a greater extent than did the development of the placentae. It was in no way equal to the needs brought about by the rapid fetal growth.

Scattergrams of the total quantity of thiamine present in carcass tissue at the different stages of pregnancy indicated no consistent trend; apparently the thiamine stores of the carcass remained much the same when the diet was adequate.

The total placental tissue being produced increased in weight gradually, being approximately 2 grams by the 13th or 14th day of pregnancy and 7.5 grams at parturition. The concentration of thiamine in such tissue varied from a low value of 2.0 micrograms to a high value of 4.1 micrograms per gram of moist tissue. Placental tissue was somewhat richer in thiamine than the maternal carcass but contained only a fraction as much as that found in hepatic tissue. It is apparent from Figures 45 and 46 that thiamine requirements were not greatly altered by the development of this tissue.

Fetal growth exceeded that of the placentae by approximately eight times. The total tissue present on the 13th day of pregnancy averaged 1.3 grams per female. The newborn

litters weighed approximately 55 grams. The quantity of thiamine present in fetal tissue increased slowly with age and reached a concentration of 3.0 micrograms of B_1 per gram of moist tissue at parturition. This concentration meant that the average litter of young at birth contained approximately 165 micrograms of thiamine. It was the rapid development of this tissue, especially during the last 4 days of gestation, which was responsible for the major increase in thiamine needs of the rat during pregnancy.

The rapid transfer of thiamine to the developing fetuses, the accumulation of greater amounts of thiamine in maternal hepatic tissue, and the gradual growth and deposition of thiamine in the placentae have all contributed to the total requirement of B_1 during reproduction in the rat. A summary of such findings has been depicted in Figure 46. This graph illustrates the daily thiamine need for reproduction at various stages of the gestation period which can be accounted for by tissue analyses alone. The requirements suggested here are over and above those of the non-pregnant animal. These estimates also do not include possible changes in requirement which might be brought about by inferior absorption of the vitamin or greater destruction of thiamine because of the pregnant state of the animal. We have no reason to believe, however, that the pregnant animal which receives an adequate ration is less efficient in its use of thiamine.

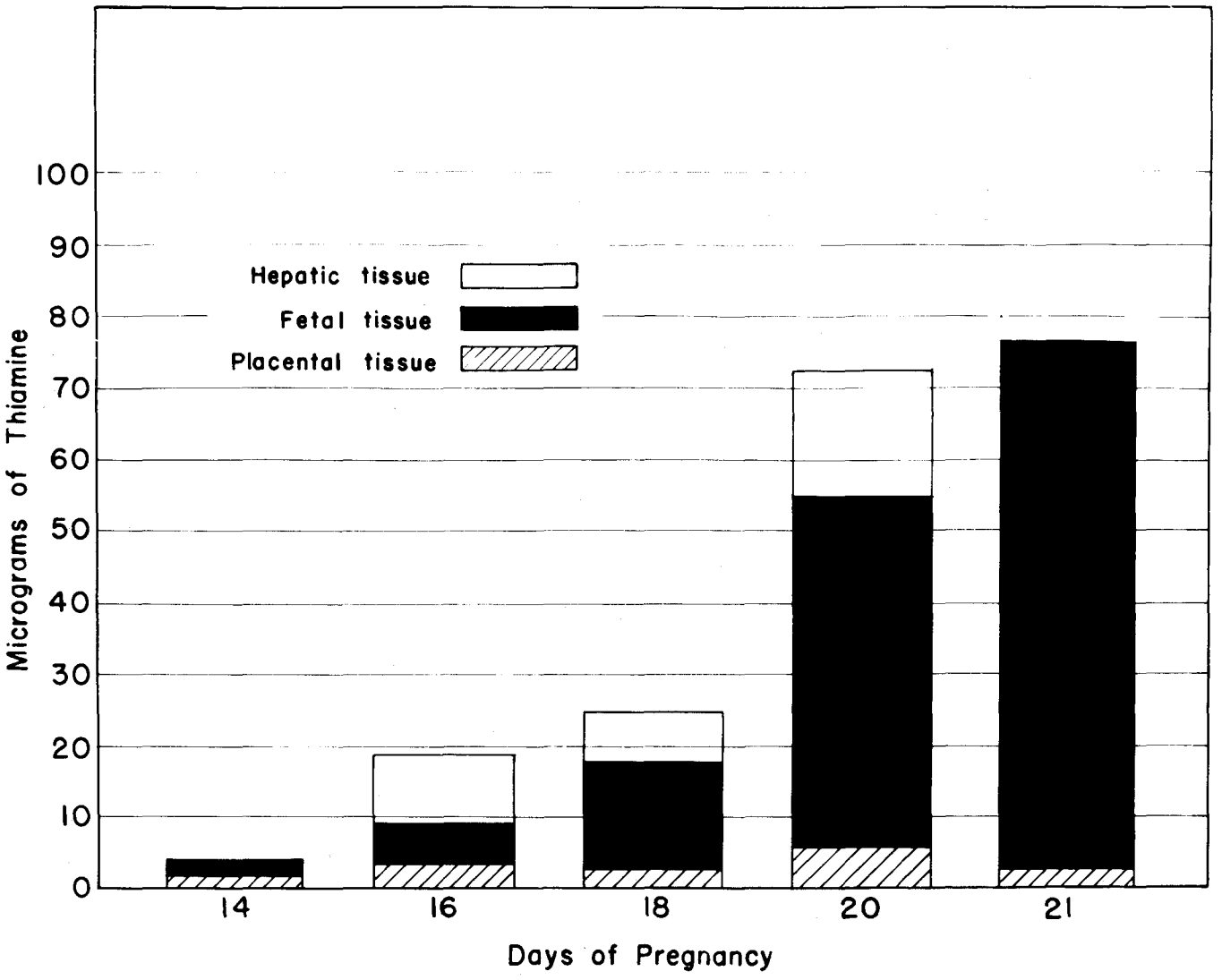

Fig. 46. Daily minimum thiamine requirements for normal reproduction in rats based on tissue analysis.

Prior to the 14th day of pregnancy there appeared very little need for additional thiamine over the amount desirable for adult animals. The quantity of thiamine deposited in embryonic and placental tissues between the 13th and 14th days of pregnancy indicated a 4 microgram need at this time. Similarly, differences in the amount of thiamine present in such tissues between the 15th and 16th days of pregnancy showed a greater demand for the vitamin; at this time also the maternal liver increased in thiamine stores thus raising the optimum thiamine intake. By the 16th day of pregnancy the total tissue changes, with respect to thiamine stores, indicated that the B_1 requirement had been enlarged to 20 micrograms. Over half of this amount was due to the increased deposition of thiamine in the liver of the mother. On the 18th day of pregnancy the total amount of thiamine needed for reproduction was still greater, approximately 25 micrograms. The very rapid growth in fetal tissue which occurred between the 18th and the final day of pregnancy augmented the thiamine need to 72 micrograms for the 20th day and 76 micrograms for the final day.

It was interesting that a large fraction of the thiamine requirement during the 20th day of pregnancy was due to the accumulation of thiamine in the liver of the female. However, there was no further increase in hepatic stores of thiamine during the final two days of pregnancy.

Many nutrition workers have heretofore considered that the vitamin needs of the rat are generally greater during the second half of pregnancy, without having a more accurate estimate of just how much greater these requirements may be. The data presented in this study indicate that no such generalizations should be made for the thiamine requirement of the rat during reproduction. In this animal, which has a very short gestation period, i.e., 22 days, and which produces a very large mass of tissue, approximately equal to 1/3 the weight of the female, the demands are quite different. Minor amounts of extra thiamine are needed prior to the 18th day of pregnancy. Between the 20th day and parturition the thiamine requirements are markedly increased. Unless some depletion of maternal tissues is to occur, it appears that at least 75 micrograms of thiamine are needed above that of the non-pregnant adult animal for satisfactory reproduction.

While these findings present evidence for unexpectedly high demands for thiamine during the last 4 days of pregnancy for the rat, it should be mentioned that the demands in human reproduction would not be expected to be acute. The much longer period of pregnancy, the more gradual growth of the offspring, and its relative size should mean a proportionately smaller need for thiamine in human reproduction.

Metabolism Experiments Conducted on Pregnant and Non-pregnant Rats as a Means of Determining Thiamine Requirements for Reproduction

Before discussing the results of the metabolism studies the writer wishes to relate some observations made while these studies were in progress. At the beginning of this portion of the experiment it was anticipated that there might be some problem due to scattering of the dry stock ration. No significant error was introduced due to spillage of food. The animals consumed quantities of ration equal to those observed for stock females housed in their regular cages. The one-inch opening of the covers of the food cups permitted the animals to eat freely and at the same time prevented them from getting food into the cage. The urine was voided directly onto the pyrex plate and due to the small urine volume and the large surface of the plate, the excreta dried rapidly. There appeared to be little chance for bacterial growth. It had been found by preliminary trials that toluene volatilized rapidly due to the large surface exposed and thus it was unsatisfactory as a possible preservative. The magnitude of the excretion of vitamins, the rather constant excretion of thiamine per individual animal, and the agreement noted between animals consuming the same amount of food have led the writer to believe that the collection procedure was satisfactory.

Immediately following the close of each metabolism period the surplus food was weighed, the cages were rinsed several times and the urine samples were diluted to 250 millliliters. It was found much more satisfactory to filter the urine through loose cotton than through filter paper. When the latter procedure was attempted during preliminary tests the length of filtering time was greatly extended and there was some evidence of loss of vitamin activity. While it was found that a volume of 250 millliliters of water provided ample liquid for quantitative removal of the urine, it should be mentioned that the urine specimens contained sufficiently large amounts of vitamins to have allowed a much greater dilution.

It will be observed that the ten females included in the metabolism experiments consumed very much the same quantity of stock ration prior to pregnancy. See Figures 47 and 48 and Tables II and III. The approximate thiamine intake during the first six metabolism periods ranged from 140 to 160 micrograms per day. During these 24 days the food consumption was fairly constant for each animal. Following the initiation of pregnancy it was observed that the food consumption increased. During several individual metabolism periods the thiamine consumption exceeded 200 micrograms for the pregnant animals, while the control females ingested between 150 and 170 micrograms of B_1 in the majority of cases.

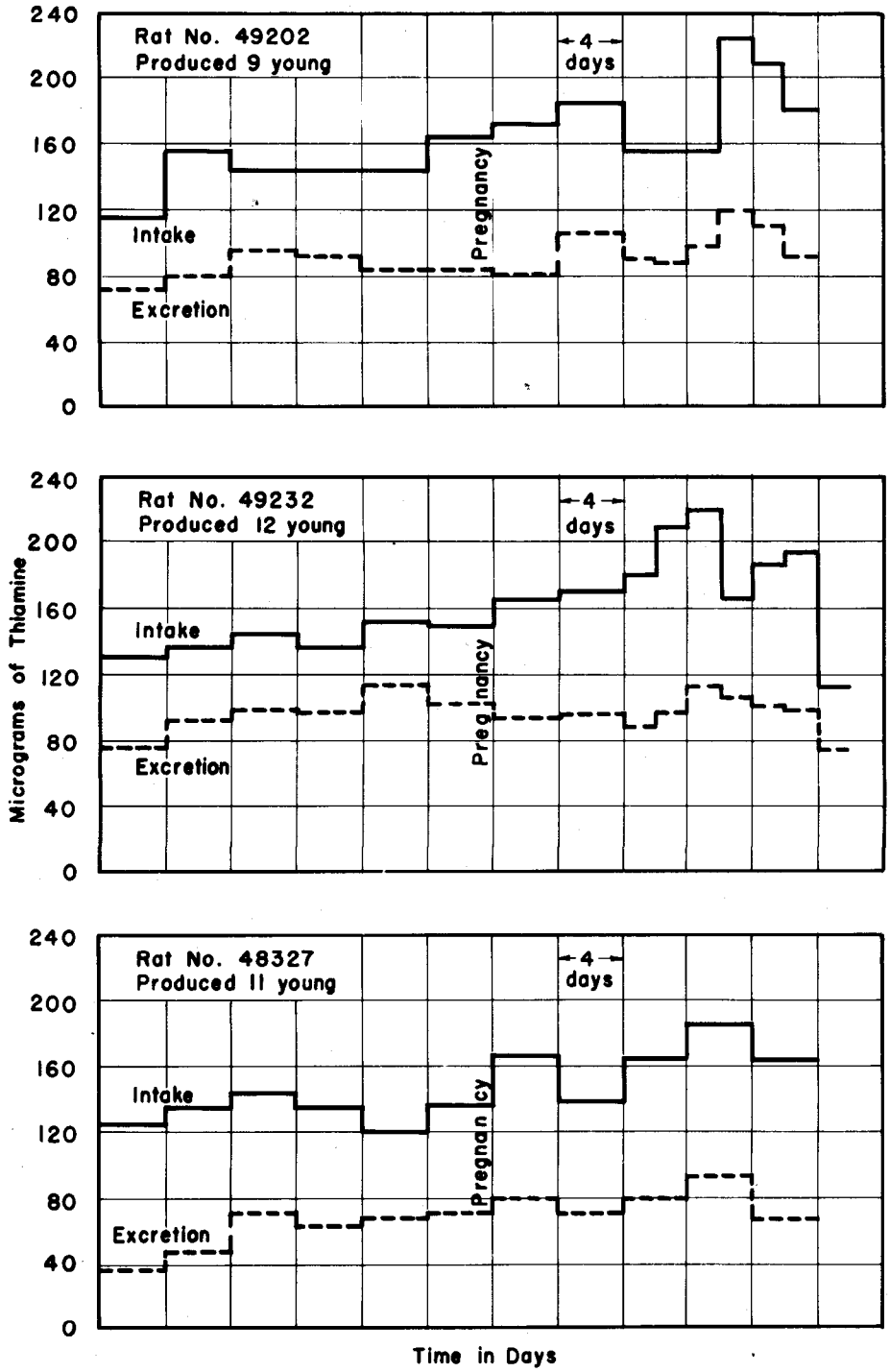

Fig. 47. The intake and urinary excretion of thiamine by pregnant rats consuming the stock ration.

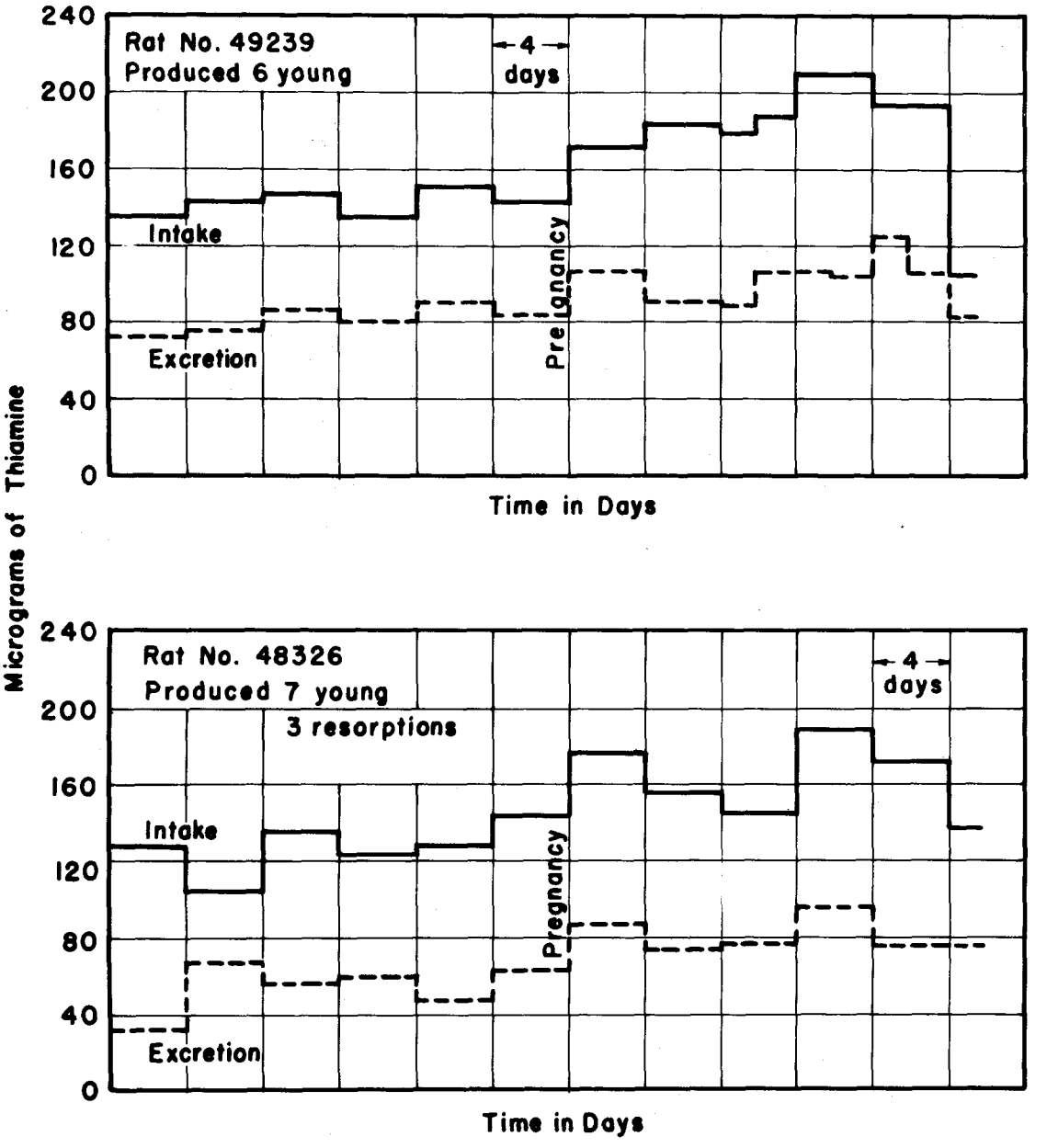

Fig. 47 (continued).

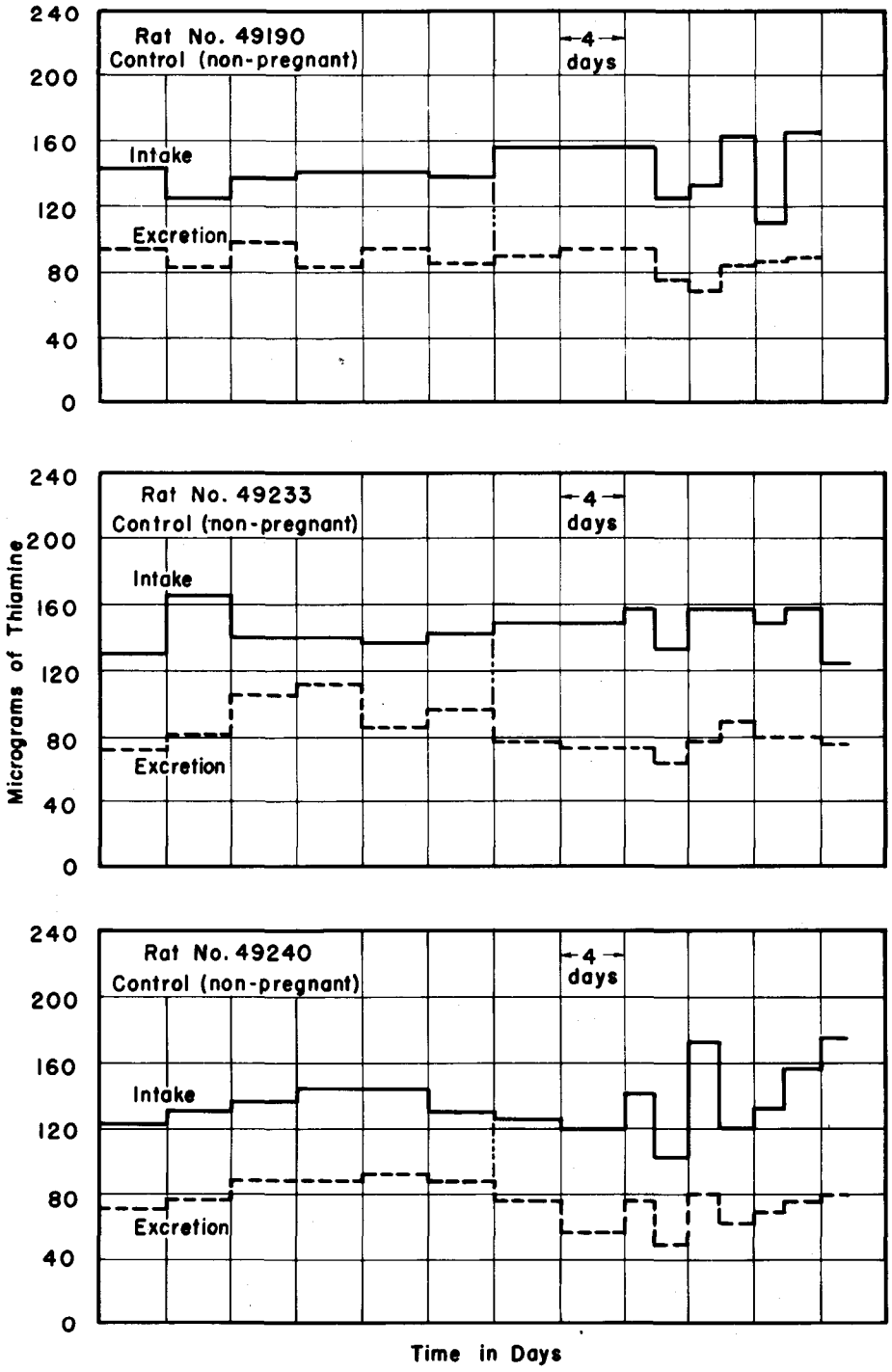

Fig. 48. The intake and urinary excretion of thiamine by non-pregnant rats consuming the stock ration.

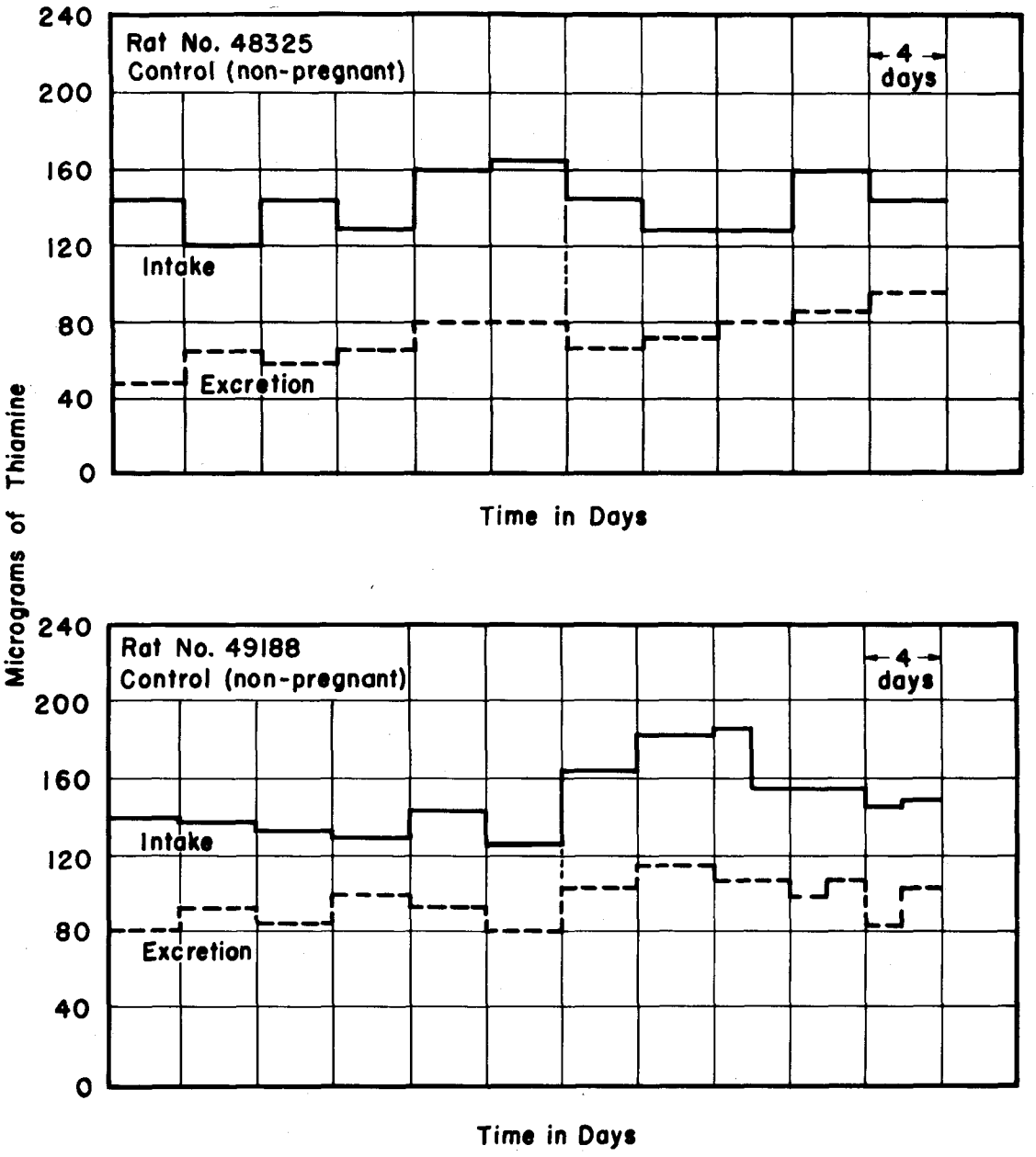

Fig. 48 (continued).

Table II

Intake, Urinary Excretion, and "Retention" of Thiamine by Pregnant Rats

Rat number	Thiamine intake per day (mcg.)	Urinary excretion per day (mcg.)	Length of period (days)	"Retention" per period (mcg.)
49239	172.8	107.9	4	259.6
	184.1	92.8	4	365.2
	180.4	91.8	2	177.2
	187.9	106.9	2	162.0
	209.2	107.9	2	202.6
	209.2	105.8	2	206.8
	195.5	125.2	2	140.6
	195.5	106.9	2	177.2
	104.6	82.1	1	22.5
				1713.7
				81.6*
	"Retention" during total gestation period =			1795.3
48326	179.5	86.8	4	370.8
	157.3	74.4	4	331.6
	145.5	76.4	4	276.4
	189.8	97.1	4	370.8
	172.1	77.2	4	379.6
	138.1	75.2	2	125.8
	"Retention" during total gestation period =			1855.2

(Continued)

*Estimated thiamine "retention" calculated from experimental data obtained during 20 or 21 days of pregnancy.

Table II (Continued)

Rat number	Thiamine intake per day (mcg.)	Urinary excretion per day (mcg.)	Length of period (days)	"Retention" per period (mcg.)
48327	167.7	80.7	4	348.0
	142.6	73.6	4	276.0
	167.7	80.7	4	348.0
	189.8	97.1	4	370.8
	167.7	71.9	4	383.2
				1726.0
				172.6*
	"Retention" during total gestation period =			1898.6
49232	165.2	93.0	4	288.8
	169.0	95.2	4	295.2
	180.4	88.5	2	183.8
	209.2	96.7	2	225.0
	218.2	113.2	2	210.0
	165.2	106.4	2	117.6
	187.9	100.9	2	174.0
	195.5	97.5	2	196.0
	112.2	74.0	1	38.2
				1728.6
				82.3*
	"Retention" during total gestation period =			1810.9
49202	172.8	82.8	4	360.0
	184.1	107.6	4	306.0
	157.6	91.5	2	132.2
	157.6	89.0	2	137.2
	157.6	99.0	2	117.2
	224.4	120.0	2	208.8
	209.2	113.1	2	192.2
	180.4	92.8	2	175.2
				1628.8
				162.8*
	"Retention" during total gestation period =			1791.6

*See note on preceding page.

Table III

Intake, Urinary Excretion, and "Retention" of Thiamine by Non-pregnant Rats

Rat number	Thiamine intake per day (mcg.)	Urinary excretion per day (mcg.)	Length of period (days)	"Retention" per period (mcg.)
49190	157.6	92.3	4	261.2
	157.6	96.7	4	243.6
	157.6	96.7	2	121.8
	127.3	77.9	2	98.8
	134.9	73.7	2	122.4
	165.2	86.8	2	156.8
	112.2	87.9	2	48.6
	165.2	89.0	2	152.4
				1205.6
				120.5*
		"Retention" during total period =		1326.1
49188	165.2	105.7	4	238.0
	184.1	117.1	4	268.0
	187.9	109.5	2	156.8
	157.6	108.2	2	98.8
	157.6	100.6	2	114.0
	157.6	109.5	2	96.2
	147.3	83.1	2	128.4
	150.1	105.7	2	88.8
				1189.0
				118.9*
		"Retention" during total period =		1307.9

(Continued)

*Estimated thiamine "retention" during interval comparable to gestation period calculated from experimental data obtained during the first 20 days.

Table III (Continued)

Rat number	Thiamine intake per day (mcg.)	Urinary excretion per day (mcg.)	Length of period (days)	"Retention" per period (mcg.)
49240	127.3	77.7	4	198.4
	119.7	59.4	4	241.2
	142.5	77.7	2	129.6
	104.6	51.8	2	105.6
	172.8	78.8	2	188.0
	119.7	63.7	2	112.0
	134.9	70.2	2	129.4
	157.6	77.7	2	159.8
	172.8	79.7	2	186.2
		"Retention" during total period =		1450.2
48325	147.6	68.9	4	314.8
	130.8	75.4	4	221.6
	130.8	80.6	4	200.8
	160.3	88.4	4	287.6
	147.6	97.2	4	201.6
				1226.4
				122.6*
		"Retention" during total period =		1349.0
49233	150.1	78.5	4	286.4
	150.1	74.0	4	304.4
	157.6	74.0	2	167.2
	134.9	65.0	2	139.8
	157.6	79.6	2	156.0
	157.6	89.7	2	135.8
	150.1	81.8	2	136.6
	157.6	82.9	2	149.4
	127.3	77.3	2	100.0
		"Retention" during total period =		1575.6

*See note on preceding page.

It was evident when the first urine analyses were completed that the stock ration provided considerable surplus thiamine. The quantity of thiamine present in the urine was high in all cases. During the seventh to final collection periods (equivalent to the gestation period of the pregnant animals) the control females excreted approximately 80 micrograms of thiamine per day. In the pregnant group the excretion was more variable. It appeared to be influenced by the increased food consumption of the animals and by the changing needs for thiamine due to the development of the fetuses.

It is well known that an appreciable excretion of thiamine by the kidney signifies that the animal is consuming an excess of this vitamin. If additional amounts of thiamine are given orally, it is not possible, however, to recover 100 per cent of the extra thiamine in the urine. A fraction of the vitamin is either destroyed before absorption can occur, escapes absorption, or is destroyed after the vitamin is in the blood stream. In the present study it has been assumed that pregnancy does not alter the efficiency of absorption of thiamine or its destruction. When comparisons were made of the "retention" of thiamine (difference between the intake and urinary excretion) by pregnant and non-pregnant animals, it was observed that B_1 needs were increased during pregnancy.

It will be noted from Tables II and III that the quantity of thiamine retained by the pregnant animals averaged 1831

micrograms for the 22-day gestation period. The virgin animals had an average "retention" of 1398 micrograms during the same interval of time. This difference in storage, presumably due to the increased needs during pregnancy, is equal to 431 micrograms.

If the first 20 days of pregnancy are divided into five 4-day metabolism periods and the "retention" of thiamine is compared for the two groups of animals, it will be found that the pregnant females retained greater proportions of thiamine during all five collection periods. However, the "retention" was greatest during the fifth study, i.e., between the 17th and 21st days of pregnancy. These data are given in Table IV.

Table IV

The "Retention" of Thiamine by Pregnant and Non-pregnant Rats

Time of pregnancy	"Retention" of thiamine		"Retention" due to reproduction
	Pregnant animals	Non-pregnant animals	
	mcg.	mcg.	mcg.
First period (Day 1-5)	325	260	65
Second period (Day 5-9)	315	256	59
Third period (Day 9-13)	330	244	86
Fourth period (Day 13-17)	361	274	87
Fifth period (Day 17-21)	364	239	125

The estimated need of 125 micrograms of thiamine noted during this late stage of pregnancy agreed closely with the requirement suggested from former tissue analyses. During the 17th through the 21st days of pregnancy tissue analyses accounted for an increased storage of 110 micrograms of thiamine.

While the increased storage of thiamine from the 1st to the 17th days of pregnancy indicated by the metabolism procedure exceeded those estimated from tissue analyses alone, the suggested requirements are still relatively small, i.e., 15-22 micrograms per day.

It was to be expected that the second method of estimating thiamine requirements during pregnancy would indicate a somewhat higher thiamine need than was predicted by direct analyses of the tissues, since the metabolism technique included any inefficiencies in the absorption of thiamine or in its transfer to the developing tissues.

The occurrence of thiamine in the tissues of the 10 females included in the metabolism studies is given in Table V. The data confirm our earlier findings that there is an accumulation of thiamine in the liver of the female during pregnancy. This increase in B_1 in the liver averaged 45 micrograms for the five metabolism animals, an amount which agreed closely with that observed in the group of animals sacrificed at various intervals during pregnancy.

Table V

Thiamine Content of Tissues of Rats Included in the Metabolism Studies

Rat number	Thiamine in tissues								
	Carcass			Liver			Fetuses		
	Wt. (gm.)	Per gm. mcg.	Total mcg.	Wt. (gm.)	Per gm. mcg.	Total mcg.	Wt. (gm.)	Per gm. mcg.	Total mcg.
Pregnant animals									
49202	116.2	1.7	193.3	7.8	13.4	105.4	43.6	3.2	138.5
49232	118.8	1.7	196.2	9.5	12.8	121.2	56.0	3.1	172.6
49239	-	-	-	8.5	14.4	122.4	32.8	3.2	107.5
48327	131.3	1.9	244.0	8.6	12.5	107.0	52.6	2.9	150.0
48326	135.2	1.9	253.0	8.9	14.8	132.0	36.4	2.9	105.0
Non-pregnant animals									
49188	110.1	1.9	208.8	6.7	10.3	69.0	-	-	-
49190	105.3	2.0	210.4	6.5	11.2	72.5	-	-	-
49233	107.7	1.9	200.6	6.2	11.0	68.4	-	-	-
49240	100.3	1.9	194.2	6.6	10.5	69.4	-	-	-
48325	118.7	1.8	213.6	7.2	10.0	72.0	-	-	-

As was found earlier, the concentration of thiamine in the carcass remained unchanged.

The females studied in the metabolism experiments were about 92 days of age when they were sacrificed at the close of their first pregnancies. At this time the carcass tissue weighed approximately 116 grams and the quantity of thiamine present in the tissue averaged 212 micrograms. When these values are compared with older animals, i.e., those included in the first experiment of this study (Table I), it will be observed that the weight of the carcass of such an animal was considerably greater and that the total amount of thiamine present in the carcass was increased. Additional data regarding the difference in size of the carcass tissue and the quantity of thiamine present in the female rat at the initiation of first and second pregnancy are given by Bear (1950, p. 44) and Spivy (1947, p. 41). These differences bring out an interesting and important point regarding total thiamine needs during pregnancy. The rat is considered mature at approximately 7 weeks of age, and first litter young are often born when the animals are 10 to 11 weeks of age, although the female is still growing. Her demands for thiamine for increased body growth are still continuing. While females during first and second pregnancies require the same amount of thiamine for alterations in hepatic stores due to pregnancy and for the development of the placentae and

fetuses, the younger animals must receive a higher dietary intake of thiamine for satisfactory reproduction. Possibly this point has not been sufficiently stressed with regard to human reproduction.

SUMMARY AND CONCLUSIONS

The thiamine requirement of the albino rat for reproduction has been investigated by two procedures. The quantity of B_1 present in maternal and fetal tissues has been determined at thirteen intervals during the gestation period and the minimum daily needs for thiamine imposed by pregnancy were established by comparing the amount of vitamin present in the tissues from day to day. This experimental approach has revealed several interesting facts concerning the need for thiamine during reproduction.

Maternal hepatic tissue increased in thiamine content during the course of pregnancy, thus raising the dietary need for this vitamin. Placental development was responsible for only minor increases in the B_1 requirement. Fetal growth, which was especially rapid between the eighteenth and final days of pregnancy, created the greatest demand for thiamine and brought the daily need to at least 76 micrograms at the close of pregnancy. Total thiamine needs for reproduction accounted for by tissue analyses alone averaged 230 micrograms for the entire gestation period. Of this amount 165 micrograms were traceable to the development of the fetuses, 26 for the placentae, and approximately 40 micrograms for the accumulation of thiamine in the liver of the female.

Metabolism studies were conducted on five pregnant stock females during the entire gestation period, and the intake and urinary excretion of thiamine of these females was compared with those of a group of non-pregnant littermates studied over a comparable interval of time. The storage of thiamine was greatest in the pregnant animals; the quantity of B_1 retained was highest between the 17th and 20th days of pregnancy. Differences in the total amount of thiamine retained by the two groups, as estimated from intake data and the quantity of thiamine excreted by the kidney, revealed that the pregnant animals needed approximately 430 micrograms of additional dietary thiamine for reproduction. This estimation of the amount of thiamine needed for reproduction allowed for some destruction of thiamine prior to absorption and for some inefficiency in absorption or transfer of the factor to the developing tissues. It therefore exceeded the requirement predicted from tissue analyses.

When data from the two experiments were studied, it was obvious that thiamine needs for reproduction in the rat are also influenced by the age and body stores of the female. The animals included in the metabolism studies were approximately 92 days of age; their carcass weights and the total amounts of thiamine stored in their bodies were smaller than those of animals sacrificed for tissue analyses. During the first pregnancy such animals were still growing and requiring

thiamine for this purpose. This finding seemed of particular interest since all females in the investigation had been mated four weeks after the opening of the vaginal orifice, i.e., at the earliest time at which they were able to reproduce. These findings emphasize that a thiamine intake sufficient for satisfactory reproduction in an older animal will not necessarily be adequate for the younger rat equally well fed.

PART III

RIBOFLAVIN REQUIREMENTS FOR REPRODUCTION

The importance of riboflavin in the reproduction of rats has been forceably demonstrated by the work of Warkany et al. (1943), who have shown that insufficient riboflavin between the thirteenth and fifteenth days of pregnancy resulted in an abnormal development of the skeleton of the embryo. The abnormality seemed to be due to the inability of certain mesodermal cells to make the transition from mesenchyme to the membranous structures which would normally become cartilaginous and osseous skeleton. Since the formation of cartilage begins about the fourteenth or fifteenth days of gestation in the rat, it is at this time that the requirement for riboflavin is most crucial in preventing abnormalities. These findings have been confirmed by Giroud and Boisselot (1947).

It is now recognized that more severe depletion of riboflavin results in anestrus (Coward et al., 1942), while less severe withdrawal of the vitamin permits the development of young which appear normal although their stores of riboflavin are not equal to those of healthy stock young (Williams, 1947).

It might be expected that these observations would have stimulated greater interest in the establishment of quantitative needs of the rat for riboflavin, especially during the

reproductive cycle. Very few data concerning this point have been published. Giroud and Boisselot have included 120 mcg. of riboflavin per day in the ration of pregnant animals and have found the young to be free of all deformities, while Warkany and his group noted that as little as 80 mcg. per day was sufficient for the development of normal-appearing young. In spite of the evidence that the riboflavin intake of the rat should be increased during pregnancy, nutrition workers have continued to select amounts of riboflavin ranging from 50 to 200 mcg. per day in formulating synthetic rations planned for studies on reproduction.

It would appear from the data in the literature that additional emphasis on the quantitative riboflavin needs of the rat during reproduction is desirable.

There are few accounts of manifestations of riboflavin deficiency in women during pregnancy. One of this small number is reported by Braun (1945), who has contributed some exceedingly interesting information concerning the value of this vitamin during human pregnancy. Of 900 pregnant Jewish women studied in Palestine, Braun observed that 190 showed deficiency signs which he attributed to too little riboflavin. These symptoms, including glossitis, cheilosis, and angular stomatitis, were more pronounced during the latter half of pregnancy and disappeared soon after parturition. Administration of either pure riboflavin or yeast alleviated the

symptoms. Dietary records of the vitamin intake of these women indicated that they were ingesting less than 1.3 mg. of riboflavin daily. Excretion values also indicated the presence of a deficiency since the quantity of riboflavin present in the urine was roughly one fourth that of healthy pregnant women. In a more recent publication Brzezenski, Bromberg and Braun (1947) correlated a higher incidence of prematurity, prenatal deaths, and less satisfactory gains in weight of infants during the first few weeks of life, with low riboflavin excretion values of the mother.

In this country Oldham et al. (1947) studied pregnant women on a known intake of 1.75 mg. riboflavin per day and observed that the subjects were excreting an average of 300 mcg. of the vitamin in the urine. If this dietary intake is sufficient, as is indicated from the excretion figures, the present recommended intake for riboflavin for women during pregnancy is very liberal. (See National Research Council, 1948.)

Today this allowance for pregnancy is 2.5 mg. per day, which is more than 50 per cent above that of the moderately active non-pregnant adult. Possibly this allowance is advisable in view of alterations in food consumption encountered during pregnancy as a result of nausea, restrictions due to excessive weight gain, and so forth. It is interesting, however, that the recommended allowance for riboflavin is

1.6 times that of thiamine for the woman during pregnancy.

In the present study more information was sought concerning the quantitative riboflavin needs during reproduction in rats. The two techniques used for the thiamine studies have been continued for information about riboflavin, i.e., an analysis of the deposition of riboflavin in the maternal and fetal tissues at various stages of pregnancy and a determination of the ingestion and excretion of riboflavin by pregnant and non-pregnant littermates fed a satisfactory stock ration which provided at least 100 mcg. of the vitamin per day throughout the gestation period.

EXPERIMENTAL PROCEDURE

The tissues and urine specimens assayed for riboflavin were the same as those used in Part II, dealing with the thiamine needs of the rat during pregnancy. The details of the experimental procedure are therefore given in Part I, pages 49 to 56, and in Part II, pages 86 to 91.

Riboflavin Assay Procedure

Aliquots of the thoroughly blended tissues estimated to contain approximately 10 mcg. of riboflavin were hydrolized with 50 ml. of 0.1N H_2SO_4 for 20 minutes at 15 pounds of pressure. When the samples were cool, sufficient 2.5M sodium acetate was added to bring the pH to 4.0. Congo red paper was used as the indicator. The preparations were filtered through Whatman paper until clear and the samples were diluted to 100 ml. Suitable aliquots were removed, neutralized to pH 6.6-6.8 with NaOH, and diluted to a final volume of 100 ml. Analyses for riboflavin were made by the microbiological method described by Snell and Strong (1939). Lactobacillus casei Number 7469* was the test organism in all cases. The media used throughout the major portion was that proposed by

*American Type Culture Collection, Washington, D. C.

Landy and Dicken (1942). However, during the final series of assays difficulty was encountered in getting acceptable standard curves for riboflavin. Concentrations of riboflavin in the tissues of 10 animals (Table X) were obtained through the use of a less synthetic basal medium published by Snell and Strong. The excellent agreement between these values and those of other animals has led the writer to believe that the nutritional requirements of the organism had changed in some manner and that the more synthetic medium was no longer satisfactory.

Urine specimens containing riboflavin in the free form were diluted to suitable volumes, neutralized to pH 6.6-6.8 and assayed directly.

RESULTS AND DISCUSSION

Deposition of Riboflavin in Maternal and Fetal Tissues as a Means of Determining Riboflavin Requirements for Reproduction

The quantity of riboflavin present in fetal and maternal tissues at various stages of pregnancy has revealed several interesting points concerning the riboflavin requirement of the rat. These findings have been summarized in Table VI and Figures 49 and 50. It will be apparent from the data that the requirement for this vitamin is not a constant one throughout pregnancy, that it increases gradually during the early portion of the gestation period and that it rises abruptly due to the rapid fetal growth during the last four days of reproduction. Development of placental tissue accounted for a very minor portion of the extra vitamin requirement; in fact, at parturition placental tissue contained less than 8 per cent as much riboflavin as that present in the fetuses. The concentration of riboflavin per gram of placental tissue remained remarkably constant throughout the entire gestation period. Fetal tissue, on the other hand, gradually increased in riboflavin concentration, from 0.4 mcg. per gm. on the 13th day of pregnancy to 2.5 mcg. at parturition.

Table VI

Concentration of Riboflavin in Fetal and Maternal Tissues

Period of pregnancy (days)	Rat number	Riboflavin in tissue: Carcass Per gm. mcg.	Carcass Total mcg.	Liver Per gm. mcg.	Liver Total mcg.	Placentae Per gm. mcg.	Placentae Total mcg.	Fetuses No.	Fetuses Per gm. mcg.	Fetuses Total mcg.
Full term	47718	2.9	416	22.5	200	-	-	10	2.9	139.3
	47721	2.3	328	21.0	205	-	-	12	2.4	137.5
	47751	2.5	370	26.4	261	-	-	11	2.3	131.9
21	47717	2.7	404	27.8	242	1.5	10.2	10	2.5	115.3
	47749	2.4	364	27.2	253	1.4	10.7	11	2.0	98.4
	47799	3.0	402	28.4	244	1.4	11.3	10	2.1	110.7
20	47750	2.4	393	27.2	280	1.5	9.6	10	1.2	42.5
	47722	2.7	427	27.0	257	1.4	11.2	13	1.6	59.1
	48640	2.4	360	27.4	296	1.1	7.1	11	1.8	56.1
19	47798	2.6	395	29.6	288	1.2	9.1	11	1.4	25.4
	47902	2.4	354	28.9	272	1.3	7.9	9	1.3	26.0
	47895	2.7	400	28.4	278	1.3	7.6	9	1.5	23.5
18	47827	2.5	337	25.8	222	1.2	6.7	10	0.8	11.5
	47851	2.9	441	24.3	245	1.5	8.1	10	0.8	11.4
	47903	2.9	422	26.5	228	1.3	6.5	10	1.1	13.9

(Continued)

Table VI (Continued)

Period of pregnancy (days)	Rat number	Riboflavin in tissues								
		Carcass		Liver		Placentae		Fetuses		
		Per gm. mcg.	Total mcg.	Per gm. mcg.	Total mcg.	Per gm. mcg.	Total mcg.	No.	Per gm. mcg.	Total mcg.
17	47912	2.5	359	29.4	262	1.4	6.4	10	0.8	5.5
	47854	2.7	383	27.0	234	1.3	7.2	11	0.9	7.9
	48472	2.6	402	26.6	279	1.2	4.8	9	0.9	5.5
16	47796	2.7	421	22.7	227	0.7	4.0	10	0.3	1.6
	47881	2.6	410	27.4	269	1.2	5.1	12	0.6	3.2
	48505	2.8	444	20.3	242	1.2	6.6	12	0.6	3.8
15	47927	2.8	405	22.9	227	0.9	3.4	11	0.7	1.7
	47913	2.8	391	23.4	225	0.8	2.7	11	0.7	1.7
	48513	3.0	409	24.2	242	0.9	3.5	12	0.8	2.7
14	47936	2.8	416	25.4	254	1.2	2.1	9	1.1	1.8
	47928	2.8	414	24.9	209	1.4	2.2	12	0.6	0.9
	47909	2.5	353	23.5	230	1.8	4.4	9	0.5	1.3
13	47828	2.5	350	22.1	212	1.0	2.0	10	0.2	0.2
	47896	2.2	317	27.6	243	1.0	2.7	13	0.3	0.5
	47777	2.3	397	29.3	279	1.8	2.7	12	0.6	0.7

(Continued)

Table VI (Continued)

Period of pregnancy (days)	Rat number	Riboflavin in tissues						
		Carcass		Liver		Uterus and its contents*		
		Per gm. mcg.	Total mcg.	Per gm. mcg.	Total mcg.	No. in litter	Per gm. mcg.	Total mcg.
12	47911	2.8	407	27.5	292	13	1.1	5.6
	47935	2.9	418	27.9	260	14	1.0	5.2
	47853	2.4	397	25.1	243	14	1.1	5.4
11	47925	2.8	405	23.4	234	11	1.1	3.3
	47882	2.7	422	24.0	240	15	1.1	4.1
	47937	2.9	421	25.2	234	13	1.4	3.8
10	48463	2.8	381	27.3	232	12	1.7	4.5
	48464	2.5	357	27.9	276	12	1.2	3.1
	48644	2.4	338	28.3	255	12	1.1	2.7
6	47852	2.4	327	29.4	235	-	0.7	1.3
	47778	2.8	379	24.9	207	-	1.0	1.5
	48646	2.6	344	28.2	253	-	0.9	1.4
Non-pregnant	47715	-	-	-	-	-	1.2	1.4
	47716	-	-	-	-	-	1.2	1.8
	47910	-	-	-	-	-	1.1	1.5

*Note difference in process prior to 13th day.

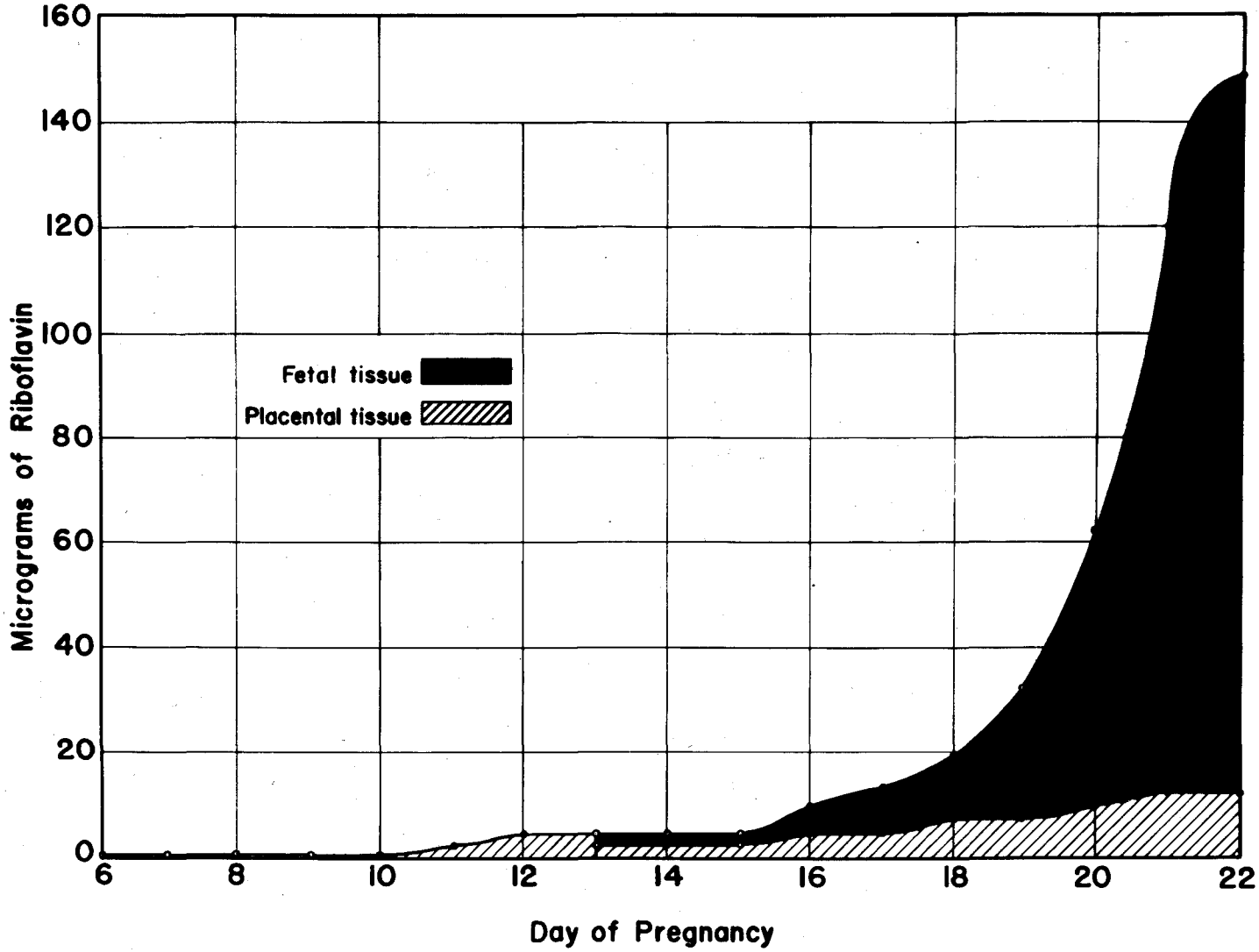

Fig. 49. Riboflavin content of placental and fetal tissue at various stages of pregnancy.

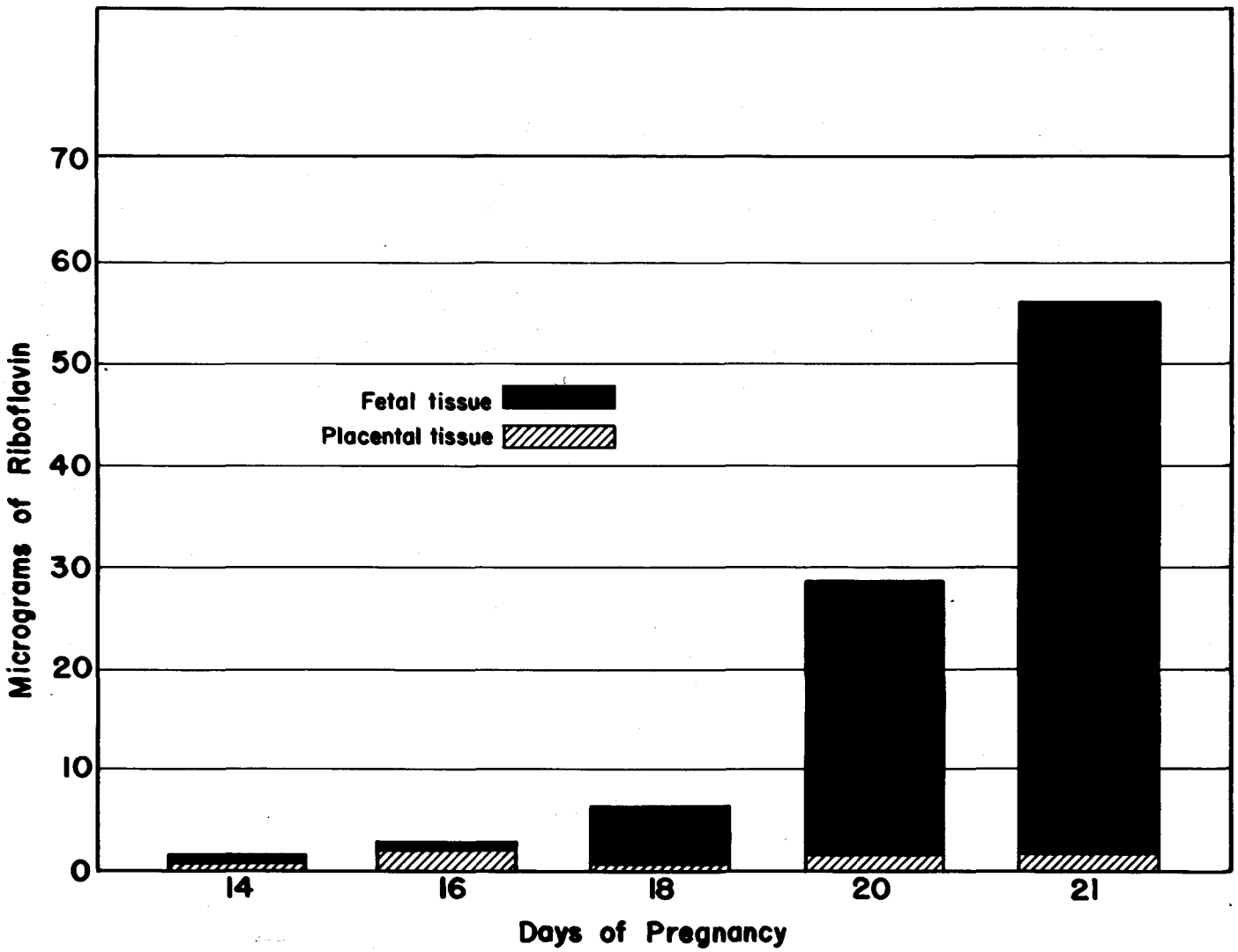

Fig. 50. Daily minimum riboflavin requirements for normal reproduction in rats, based on tissue analyses.

During that interval of the prenatal period which Warkany and his associates (1943) found critical in the development of malformations in the rat, the combined placental and fetal tissue demands were equal to from 3 to 5 mcg. of riboflavin. These findings emphasize the fact that prevention of malformations does not require particularly high ingestions of riboflavin, but that it is important for the reproducing animal to meet the adult needs regularly. During later stages of pregnancy the daily need for riboflavin is appreciably increased; at term the normal litter weighing approximately 55 grams contained 130 mcg. of the vitamin.

The riboflavin content of the maternal carcass and liver remained relatively constant throughout pregnancy when the animals had access to an adequate diet providing over 100 mcg. of the vitamin per day. Hepatic tissue contained very much greater concentrations of riboflavin than any other tissue examined; i.e., liver varied from 21 to 29 mcg. per gram; maternal carcass ranged from 2 to 3 mcg. per gram; the fetuses varied from 0.2 to 2.9 mcg.; and placental tissue averaged 0.8 to 1.5 mcg. per gram of fresh tissue. The concentration of riboflavin found in liver was observed to be approximately twice that of thiamine.

When a comparison is made between the quantity of riboflavin present in both fetal and placental tissues from one day to the next throughout pregnancy, it is observed that

there is no serious increase in riboflavin needs due to pregnancy before the 18th day (Figure 50). However, on the 20th day of the reproductive cycle this need (due to tissue changes alone) increased to 28 mcg. above that of the non-pregnant animal. By the 21st day the requirement had reached a maximum of 56 mcg. On the final day, tissue changes were again somewhat reduced, and the indicated need was more nearly equal to that of the 20th day.

Metabolism Experiments Conducted on Pregnant and Non-pregnant Rats as a Means of Determining Riboflavin Requirements for Reproduction

The consumption and urinary excretion of riboflavin have been determined for five females maintained on the customary stock diet during a 24-day period preceding pregnancy and throughout the gestation period. An equal number of virgin animals of the same age were tested as controls. The results are presented in Figures 51 and 52 and Tables VII and VIII.

Prior to pregnancy all animals ingested 73 to 105 mcg. of riboflavin daily. This amount greatly exceeded needs since a large portion of the daily intake was excreted in the urine. With the onset of pregnancy, food consumption increased and the riboflavin intake usually exceeded 100 mcg. per day; occasionally the ingestion was above 130 mcg. Virgin animals continued to select approximately the same quantity of diet.

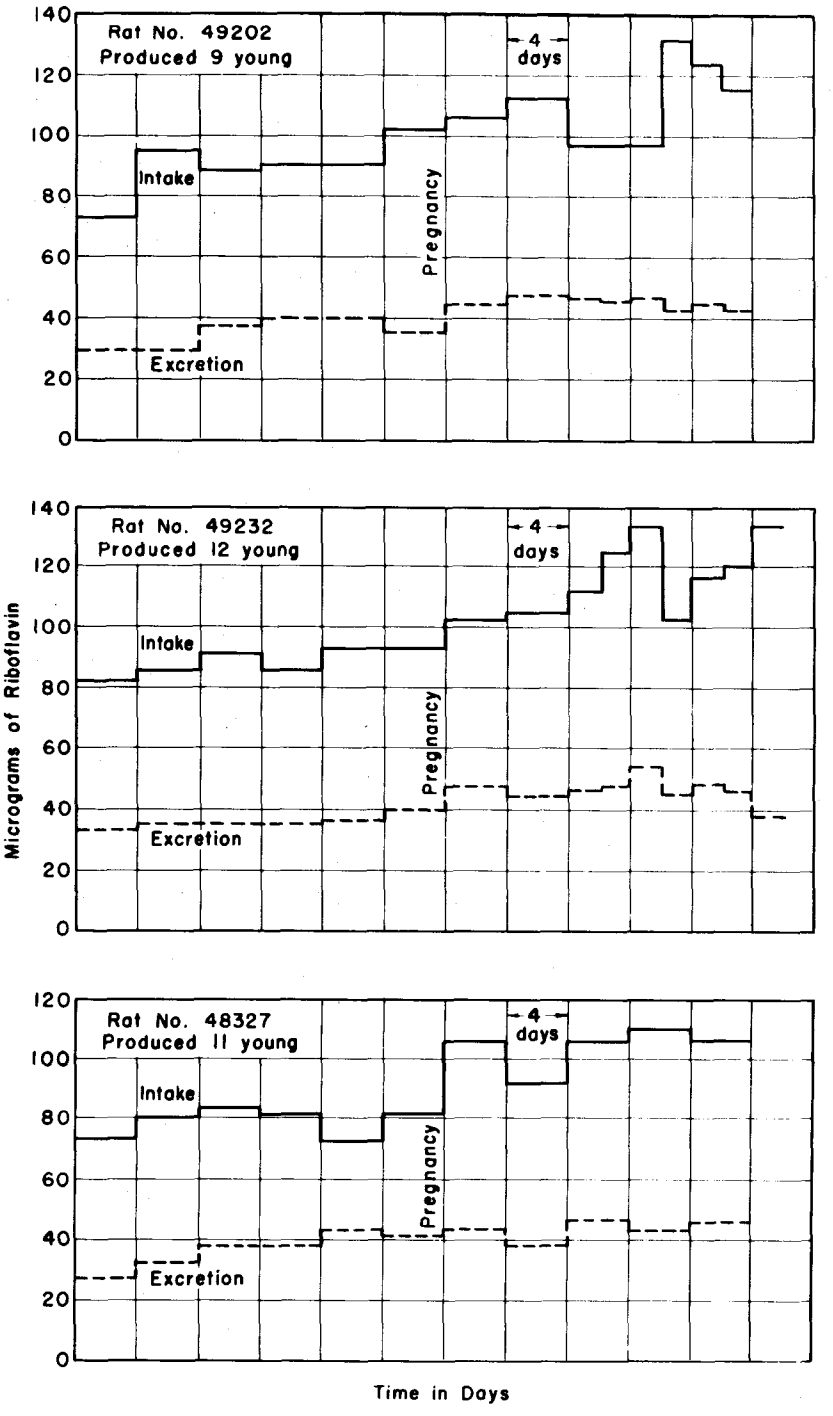

Fig. 51. The intake and urinary excretion of riboflavin by pregnant rats consuming the stock ration.

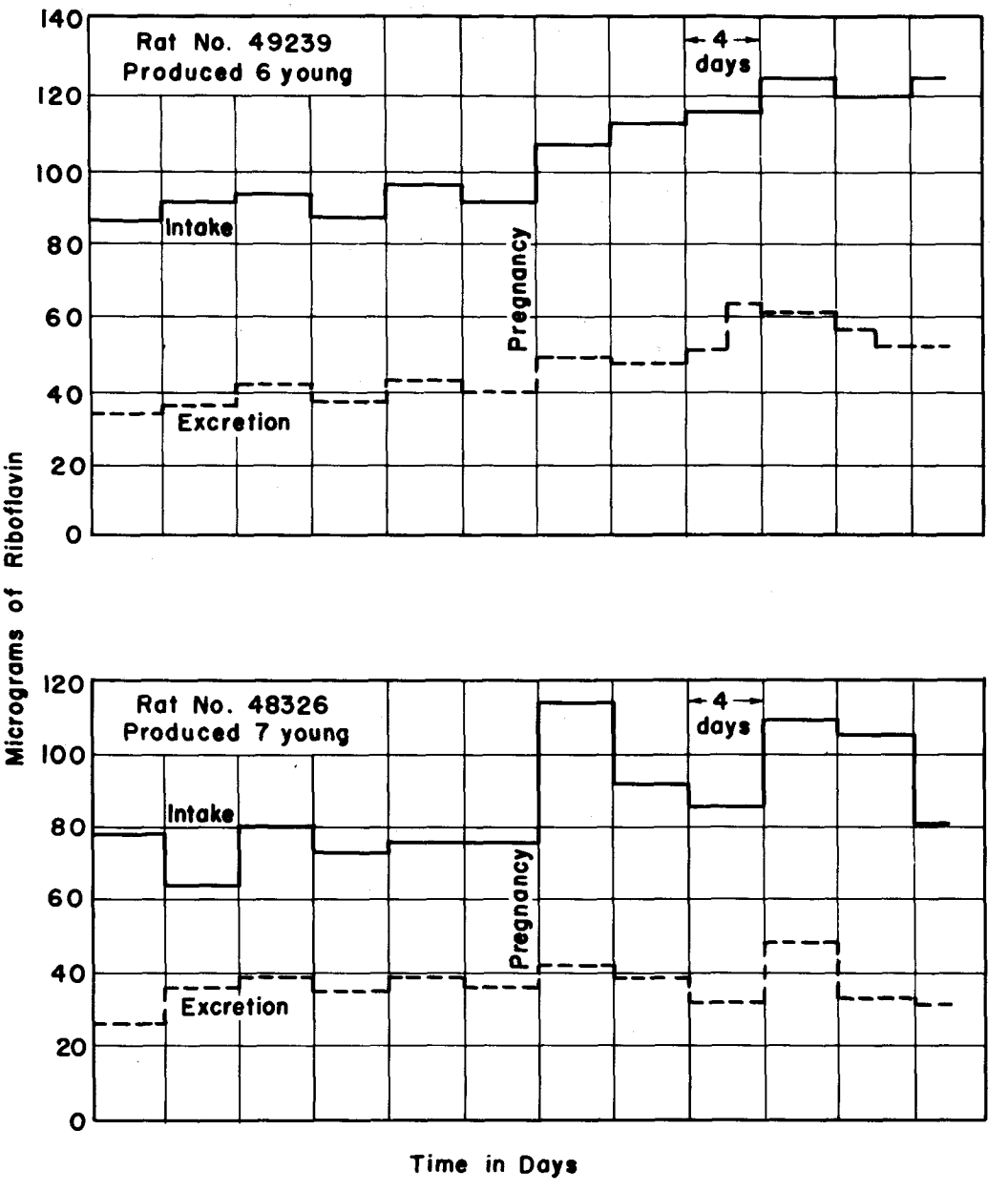

Fig. 51 (continued).

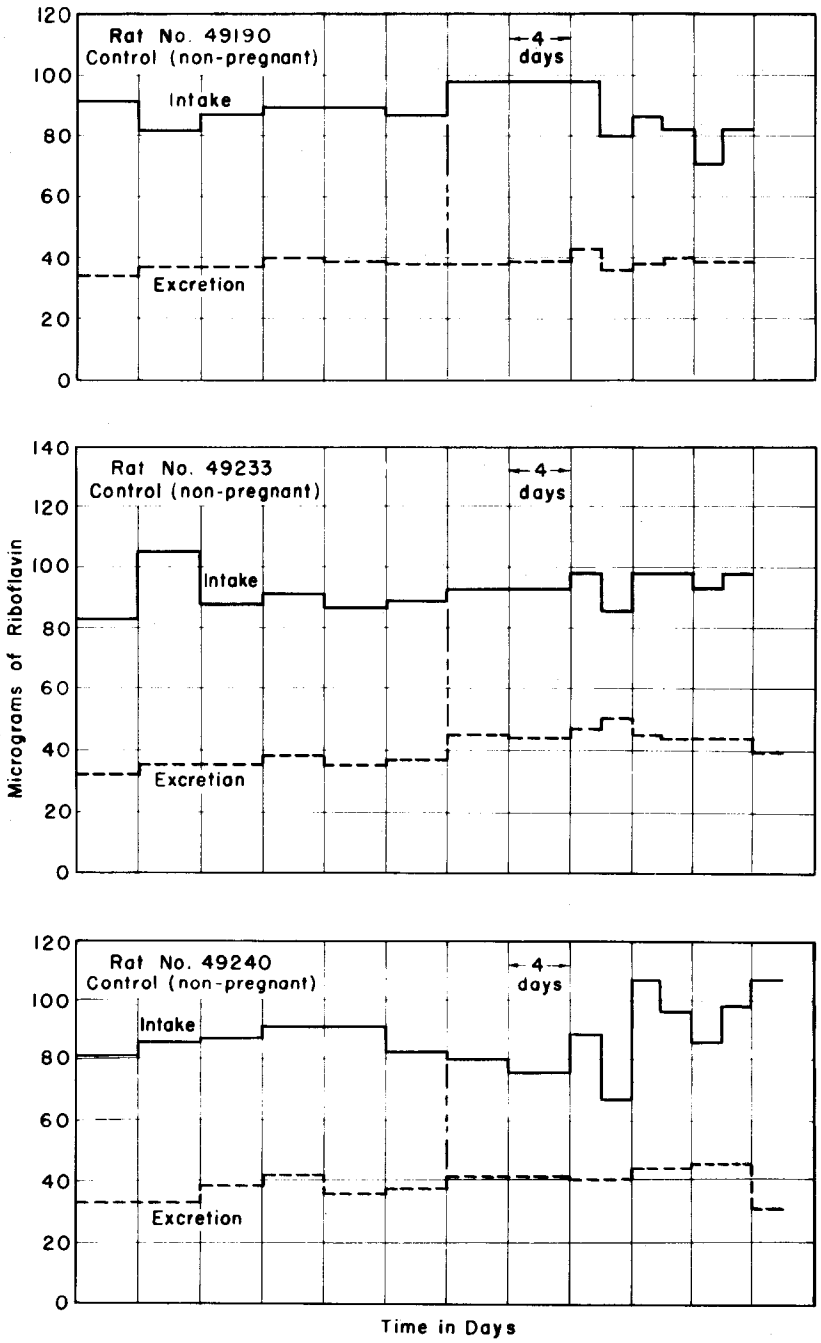

Fig. 52. The intake and urinary excretion of riboflavin by non-pregnant rats consuming the stock ration.

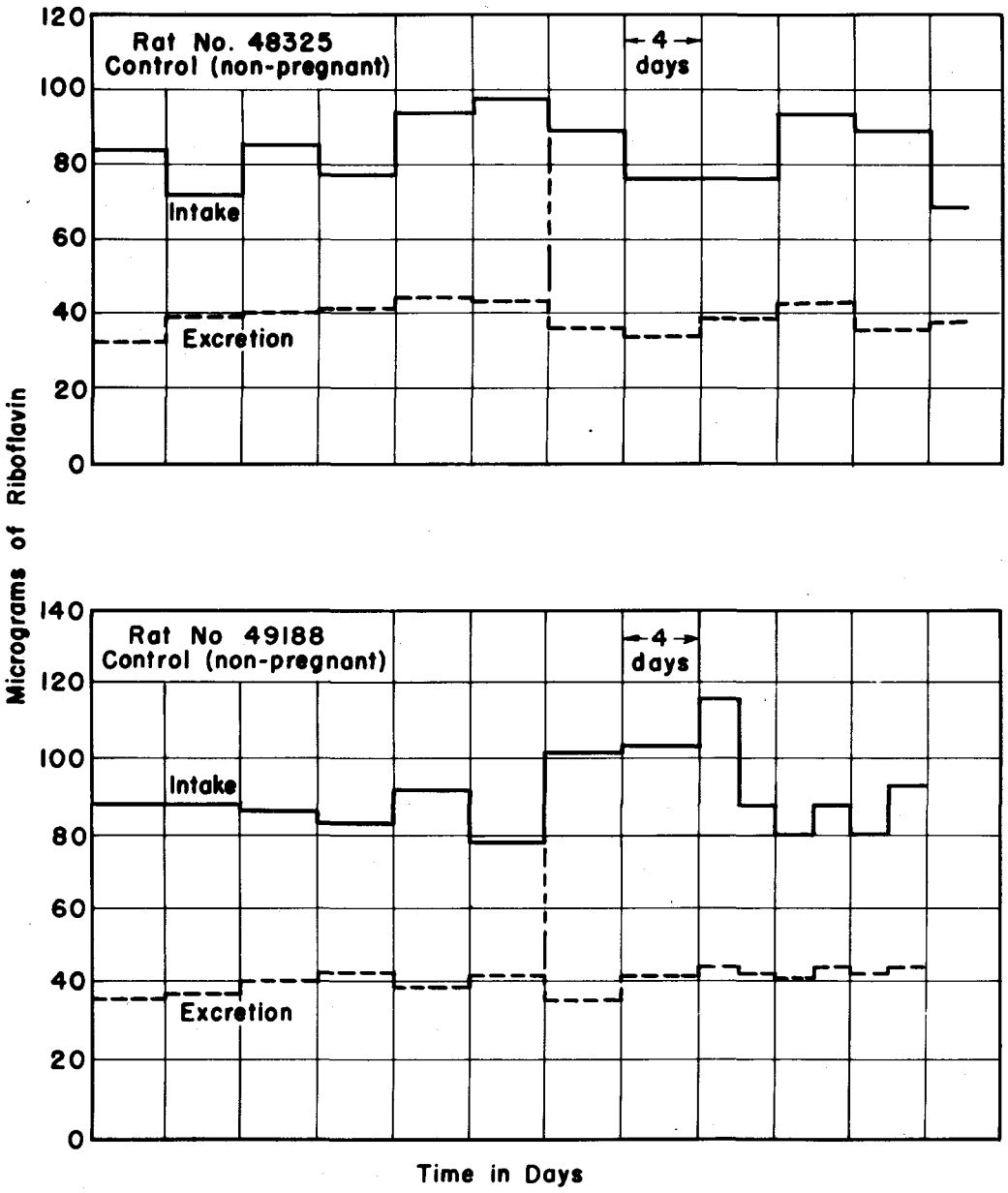

Fig. 52 (continued).

Table VII

Intake, Urinary Excretion, and "Retention" of Riboflavin by Pregnant Rats

Rat number	Riboflavin intake per day (mcg.)	Urinary excretion per day (mcg.)	Length of period (days)	"Retention" per period (mcg.)
49239	107.2	49.1	4	232.4
	113.8	48.2	4	262.4
	116.1	51.2	2	129.8
	116.1	64.3	2	103.6
	125.0	61.3	2	127.4
	125.0	61.5	2	127.0
	120.5	57.5	2	126.0
	120.5	52.5	2	136.0
	125.0	52.3	2	145.4
	"Retention" during total gestation period =			1390.0
48326	114.2	42.7	4	286.9
	92.4	39.0	4	213.6
	86.0	32.7	4	213.2
	110.2	49.3	4	243.6
	105.8	33.6	4	288.8
	81.9	32.7	2	98.4
	"Retention" during total gestation period =			1343.6

(Continued)

Table VII (Continued)

Rat	Riboflavin intake per day (mcg.)	Urinary excretion per day (mcg.)	Length of period (days)	"Retention" per period (mcg.)
48327	106.1	43.9	4	248.8
	92.4	38.1	4	217.2
	106.1	47.1	4	236.0
	110.2	43.8	4	265.6
	106.1	46.2	4	239.6
				1207.2
				120.7*
	"Retention" during total gestation period =			1327.9
49232	102.7	47.1	4	222.4
	105.0	44.9	4	240.4
	111.1	46.5	2	129.2
	124.9	47.9	2	154.0
	133.8	54.4	2	158.8
	102.7	45.1	2	115.2
	116.1	48.4	2	135.4
	120.5	46.2	2	148.6
	133.8	38.0	2	191.6
	"Retention" during total gestation period =			1495.6
49202	107.2	45.4	4	247.2
	113.8	48.2	4	262.4
	98.3	47.9	2	100.8
	98.3	46.8	2	103.0
	98.3	47.7	2	101.2
	133.8	43.0	2	181.6
	124.9	45.0	2	159.8
	116.1	43.7	2	144.8
				1300.8
				130.1*
	"Retention" during total gestation period =			1430.9

*Estimated riboflavin "retention" calculated from experimental data obtained during the first 20 days.

Table VIII

Intake, Urinary Excretion, and "Retention" of Riboflavin by Non-pregnant Rats

Rat number	Riboflavin intake per day (mcg.)	Urinary excretion per day (mcg.)	Length of period (days)	"Retention" per period (mcg.)
49190	98.3	38.1	4	240.8
	98.3	39.9	4	233.6
	98.3	43.2	2	110.2
	80.5	36.8	2	87.4
	86.0	38.3	2	95.4
	82.7	40.3	2	84.8
	71.6	39.5	2	64.2
	82.7	39.4	2	86.6
				1003.0
				100.3*
		"Retention" during total period =		1103.3
49188	102.7	35.8	4	267.6
	103.8	41.7	4	248.4
	116.1	44.2	2	143.8
	88.3	42.9	2	90.8
	80.5	41.8	2	77.4
	88.3	44.7	2	87.2
	80.5	42.3	2	76.4
	93.8	44.2	2	99.2
				1090.8
				109.1
		"Retention" during total period =		1199.9

(Continued)

*Estimated riboflavin "retention" during interval comparable to gestation period calculated from experimental data obtained during the first 20 days.

Table VIII (Continued)

Rat number	Riboflavin intake per day (mcg.)	Urinary excretion per day (mcg.)	Length of period (days)	"Retention" per period (mcg.)
49240	80.5	41.5	4	156.0
	76.1	41.6	4	138.0
	89.4	40.5	2	97.8
	67.2	40.2	2	54.0
	107.2	45.7	2	123.0
	96.0	45.9	2	100.2
	86.0	46.3	2	79.4
	98.3	46.0	2	104.6
	107.2	31.4	2	151.6
		"Retention" during total period =		964.6
48325	90.0	36.8	4	212.8
	77.9	34.9	4	172.0
	77.9	39.8	4	152.4
	94.0	43.5	4	202.0
	90.0	36.2	4	215.2
	69.8	38.4	2	62.8
		"Retention" during total period =		1017.2
49233	93.8	45.8	4	192.0
	93.8	44.6	4	196.8
	98.3	47.3	2	102.0
	86.0	50.5	2	71.0
	98.3	45.0	2	106.6
	98.3	44.3	2	108.0
	93.8	44.6	2	98.4
	98.3	44.5	2	107.6
	80.5	39.1	2	82.8
		"Retention" during total period =		1065.2

The pregnant animals, having access to between 100 and 130 mcg. of the factor per day excreted large quantities of riboflavin throughout the entire reproduction period, indicating that the diet furnished an ample supply of riboflavin at all times. In fact, it furnished a surplus.

If the quantity of riboflavin present in the urine is subtracted from that of the intake and the assumption is made that the difference reflects "retention," some comparisons can be made concerning the storage of riboflavin by pregnant and non-pregnant animals during the 22-day interval comparable to the gestation period.

Fair agreement was observed for the "retention" of riboflavin by the five pregnant animals. These values ranged from 1328 to 1495 mcg. of riboflavin for the 22-day gestation period. In certain instances this value included data from nine individual metabolism periods. In every case the quantity of riboflavin retained exceeded that of the non-pregnant animals which were storing between 965 and 1199 mcg. for the 22 days. This increased storage of riboflavin, seemingly due to the demands of pregnancy, was approximately 327 mcg. This value indicates a relatively small rise in the riboflavin requirement of the rat for satisfactory reproduction. However, as brought out from data obtained from tissue analyses, the requirement is not a stationary one.

A comparison of the "retention" of riboflavin by the two groups of animals (Table IX) during five 4-day collection periods revealed that the storage of the vitamin was highest for the pregnant animals in all cases, although the greatest difference occurred near parturition.

Table IX

The Retention of Riboflavin by Pregnant and Non-pregnant Rats

Time of pregnancy	Retention of riboflavin		Retention due to reproduction
	Pregnant animals	Non-pregnant animals	
	mcg.	mcg.	mcg.
First period (Day 1-5)	247.3	213.8	33.5
Second period (Day 5-9)	259.2	197.7	61.5
Third period (Day 9-13)	233.9	181.8	52.1
Fourth period (Day 13-17)	264.0	196.9	67.1
Fifth period (Day 17-21)	275.8	186.3	89.5

The riboflavin requirement of the rat for satisfactory reproduction, judged by "retention" values, exceeded the requirement predicted from analyses of the developing tissues, i.e., 327 mcg. versus 146 mcg. It will be clear from Table X, however, that the divergence between these two procedures was smaller than these figures would indicate. The 10 animals included in the metabolism experiments were approximately 92 days old at the conclusion of the experiment. As was

Table X

Riboflavin Content of Tissues of Rats Included in the Metabolism Studies

Rat number	Riboflavin in tissues								
	Carcass			Liver			Fetuses		
	Wt. (gm.)	Per gm. mcg.	Total mcg.	Wt. (gm.)	Per gm. mcg.	Total mcg.	Wt. (gm.)	Per gm. mcg.	Total mcg.
Pregnant animals									
49202	116.2	2.6	303	7.8	23.8	186	43.6	3.1	134
49232	118.8	2.4	284	9.5	19.1	181	56.0	2.7	152
49239	135.0	-	-	8.5	22.1	188	32.8	3.4	112
48327	131.3	2.3	305	8.6	21.2	182	52.6	3.0	160
48326	135.2	2.4	321	8.9	23.8	212	36.4	3.1	114
Non-pregnant animals									
49188	110.1	2.7	292	6.7	21.7	146	-	-	-
49190	105.3	2.7	287	6.5	24.2	158	-	-	-
49233	107.7	2.6	278	6.2	23.9	148	-	-	-
49240	100.3	2.7	266	6.6	23.6	156	-	-	-
48325	118.7	2.5	296	7.2	21.6	156	-	-	-

mentioned in Part II of the study, these animals were still growing and their body stores were continuing to change. The five females which had newly delivered their young possessed larger livers and greater total riboflavin stores in their hepatic tissue, although the concentration of the vitamin per gram of fresh tissue remained unaltered.

These differences in riboflavin stores of the body which fluctuated with the age of the animal are illustrated in Tables VI and X. The values are good proof that the young adult rat requires a higher riboflavin intake to permit satisfactory reproduction than is true for the more mature animal.

SUMMARY AND CONCLUSIONS

Two techniques have been investigated to determine more accurately the quantitative riboflavin needs of the rat during reproduction. One procedure, that of determining the deposition of riboflavin in the developing fetal and placental tissues at several intervals during pregnancy, has revealed a very rapid increase in the requirement for this factor during the last four days of pregnancy. Prior to this time, and in fact for the majority of the reproductive cycle, the full-grown rat appeared to have little need for riboflavin above that of the non-pregnant adult.

The development of the placental tissue influenced the total need for riboflavin to a negligible extent.

Metabolism studies conducted on pregnant and non-pregnant stock females maintained on a ration providing approximately 100 mcg. of riboflavin per day indicated that the diet provided a large surplus during the early portion of pregnancy and supplied some excess even during the final days of gestation, as judged by the excretion of the vitamin by the kidneys. Females receiving this ration produced large litters of active young with satisfactory birth weights. The young were well stocked with riboflavin and there was no depletion of the maternal tissues during pregnancy.

It was observed that stock females 92 days old were still accumulating body stores of riboflavin; these animals have a higher dietary need for riboflavin during reproduction than do older animals.

Thiamine and riboflavin assays of the developing fetal tissues and those of the female rat during pregnancy indicated that the need for riboflavin was less than that for thiamine. This quantitative relationship between the two vitamins is particularly interesting in view of the present recommended allowances for the two vitamins during pregnancy in women.

In the formulation of rations satisfactory for reproduction in rats, it appears that a daily intake of 100 mcg. of riboflavin is desirable during the last third of pregnancy when the demand for this vitamin reaches a maximum. This intake provides a considerable surplus during earlier phases of the pregnancy period.

Thiamine requirements exceed those for riboflavin during pregnancy in the rat, although this relationship does not follow during maintenance of the adult female. The data from the present study suggest that a daily intake of 125 mcg. of thiamine is ample for reproduction during the period of maximum need. This amount supplies a large excess of the vitamin in the first half of the gestation period.

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