SOME EMBRYOLOGICAL ASPECTS OF VITAMIN C-DEFICIENCY IN THE GUINEA PIG (*CAVIA COBAYA*)

by

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INTRODUCTION

Previous experiments performed on guinea pigs on a vitamin C-free diet indicated definite effects on the maternal tissues but little work has been done on effects on the embryo itself, so this experiment was started to see what changes are manifested in the embryo and to find, if possible, the cause of the high rate of abortion found in the pregnant scorbutic female guinea pig.

REVIEW OF LITERATURE

Harman and Prickett (1932) traced the early development of the guinea pig embryo from 11 to 20 days gestation. This was followed by a study of normal development from 21 to 35 days gestation by Harman and Dobrovolny (1933). These studies were valuable in determining at what age to put the mothers on the vitamin C-free diet and the age of embryo to choose for study. Harman and Kordisch (1945) studied the effect of varying the vitamin C-content of the diet in guinea pigs upon the blood. Harman and Traulson (1943) studied the effect of vitamin C-deficiency upon skeletal muscle. Little difference was seen in the vitamin C-content of the blood of normal animals and that of experimental animals. In this experiment as in preceding ones, the experimental guinea pigs have very little blood during the later stages of scurvy in comparison with the normals. Harman

and Gillum (1937) observed the effects on reproduction in guinea pigs fed vitamin C at different levels. Abortion or dead fetuses resulted in cases of vitamin C-deficiency. Observations on endometrial hyperplasia in guinea pigs receiving limited amounts of vitamin C were made by Saffrey, Harman, and Kramer (1937). Kramer, Harman, and Brill (1933) studied the disturbances of reproduction and ovarian changes in the guinea pig in relation to vitamin C-deficiency. Ingier (1915) found that vitamin C-deficiency in the earlier stages of pregnancy invariably resulted in premature birth or dead fetuses, but in older animals and those in the latter half of gestation gave birth to living and apparently mature offspring. Lindsey and Medes (1926) state "that male guinea pigs with mild chronic scurvy fail to reproduce". Goettsch (1930) reported similar results in the male guinea pig and also that in the females, when they were gradually deprived of vitamin C, "the oestrous rhythm was maintained regularly until the animal began to lose weight".

MATERIALS AND METHODS

Breeding Conditions

Twenty healthy young female guinea pigs were purchased from Gopher State Caviary, 862 Atlantic Street, St. Paul, Minnesota. Ten additional mature females were secured from Dr. Ibsen of the Animal Husbandry Department, Kansas State College, Manhattan,

Kansas. These were placed in special cages in an animal room with a temperature quite constant and favorable for guinea pigs. Each cage accommodated five females. Males were used for breeding only and were kept in separate cages. A female was placed with a male only when by daily inspection the vagina was found open at which time breeding was attempted. In this way the copulation age of the embryo could be known.

Feeding Conditions

The experimental animals were fed a diet consisting of:

Ingredients	g
rolled oats	350
bran	250
butter	80
cod liver oil	20
salt	10
dried skim milk	300

The milk was baked two hours at 110° C. to remove vitamin C. All other guinea pigs received pellets and grass or sprouted oats daily.

The mothers bearing experimental embryos were kept on the control diet until the 21st day of pregnancy and then placed on the vitamin C-free diet. This procedure was used to prevent early abortion of the embryos and it proved satisfactory in most cases.

Removal of Embryos

At the termination of the desired copulation age each guinea pig was killed with illuminating gas and the embryos were removed and were fixed in Bouin's fixative. The uterus and extra-embryonic tissues were also fixed in Bouin's fixative. Careful observations were made on the weight, length, and external and internal development as compared with such observations on embryos from mothers on the control diet.

In addition to this macroscopic examination of structure, a microscopic study of tissues was made. Transverse sections of skin and intestine of the embryos were prepared. The skin was from the mid-ventral line anterior to the umbilicus. These tissues were stained with Delafield's haematoxylin and triosin.

DATA AND OBSERVATIONS

General Observations

A total of 32 embryos was secured from 12 pregnant females. These embryos were at six stages of development ranging in copulation age from 27 days to 50 days. Embryos of the same copulation age were secured from both control and experimental guinea pigs. This was done so that the degree of differentiation of the tissues of the embryos of the experimental and control animals could be studied.

As a measure of general development the length and weight values of the embryos were of special interest. Table 1 shows the successive comparative values. There was some variation within litters so, for convenience, litter averages were recorded. These data indicate a progressive increase in the size of embryos but a rather striking retardation of the development of the embryos from the guinea pigs on the vitamin C-free diet.

Measurements of the diameters of the placental discs of the embryos of both experimental and control animals were made and are recorded in Table 2. The data recorded are average diameters of discs for the litter at each stage of development. A retardation in development of the discs from the vitamin C-deficient guinea pigs is evident in all stages except the 34-day stage.

The data of Tables 1 and 2 are plotted in Figs. 1 and 2, respectively.

The 27-day Embryo

At 27 days the embryos of the control guinea pig weighed on the average 0.71 g while the embryos of the experimental guinea pig weighed an average of 0.16 g. The average lengths were 1.30 cm for the embryos of the control and 1.05 cm for the embryos of the experimental. Besides being larger in size the embryos of the control showed a definitely greater degree of development. The nape flexures were marked in the embryos of

Table 1. The comparative weights and body lengths (CR) of the embryos of the control and experimental guinea pigs. The data are in terms of litter averages.

Cop. age (days)	Length (CR) in cm		Weight in g	
	Control	Experimental	Control	Experimental
27	1.7	1.0	0.71	0.16
29	2.0	1.4	0.90	0.50
34	3.7	1.6	3.6	0.60
40	4.9	2.7	8.3	2.0
42	6.4	5.0	9.8	3.7
50	8.5	5.5	40.6	8.0

Table 2. The comparative diameters of the placental discs of the control and experimental guinea pigs. The data are in terms of litter averages.

Cop. age (days)	Diameter of placental discs in cm	
	Control	Experimental
27	1.3	1.1
29	1.3	1.1
34	1.5	1.2
40	2.0	1.7
42	2.1	2.2
50	2.5	2.0

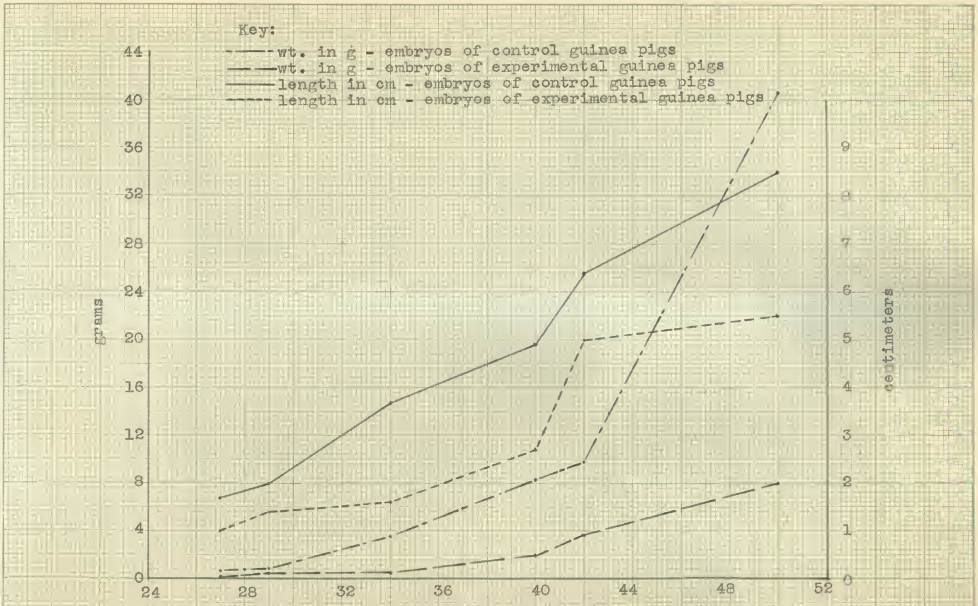

Fig. 1. The comparative weights and body lengths (CR) of embryos of the control and experimental guinea pigs.

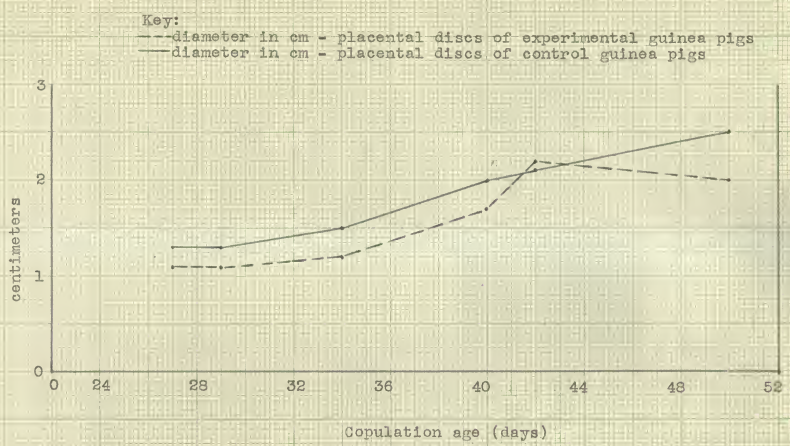

Fig. 2. The comparative diameters of the placental discs of the control and experimental guinea pigs.

the experimental and were nearly absent in the embryos of the control. The tails were distinct in the embryos of the experimental and considerably resorbed in the embryos of the control. The distal ends of the limb buds in the embryos of the experimental were flattened and grooved indicating establishment of digits, whereas the limb buds of the embryos of the control showed distinct digits. The external ear appeared as a continuous ridge in the embryos of the experimental but had developed to a fold which nearly covered the external meatus in the embryos of the control. Follicles of developing vibrissae appeared on embryos of both the control and experimental below the eyes on either side of the face but only the embryos of the control had follicles about the eyes, usually two about each eye.

Dissection showed little contrast except that internal structures were larger in the embryos of the control than in the embryos of the experimental. However a microscopic study of the skin and of the intestine showed a marked contrast in the structure of the tissues. This is demonstrated in Plate I, Figs. 3 and 4. The section of the skin of the embryos of the control showed a well defined thickened epidermal layer of cells. The underlying cells had sharply defined cell walls and large distinct, well defined nuclei. In the sections of the skin of the embryo of the experimental the outer layer was not thickened and the cell walls of the underlying cells were indistinct and rather blurred in appearance. The nuclei were small and not well defined.

A similar picture of contrast was seen in the comparison of the sections of the intestines of the embryos of experimental and control animals, Plate I, Figs. 5 and 6. In the section of the intestine of the control embryo the epithelial lining was composed of tall columnar cells with distinct cell walls and well defined nuclei. A definite basement layer was present. Also there was a distinct lumen in the intestine. The section of the intestine of the embryo of the experimental animal showed no definite cell boundaries, the nuclei seemed to be run together, and the whole epithelial lining was sloughed off.

Both the skin and the intestine of the embryos of the experimental animals did not take the cytoplasmic stain readily. Sections of these tissues were immersed in triosin one minute and a faint staining resulted while tissues from the embryos of the control stained a brilliant red after being immersed for only a few seconds in the triosin.

The 29-day Fetus

The fetuses of the control guinea pig have continued development until by the 29th day they appeared similar to the fetuses at term in flexures and general shape of the body. A single fetus from the experimental animal was secured at this stage. This fetus showed considerable nape flexure and was smaller than the fetuses of the controls as indicated in Table 1. It also appeared thinner than the fetuses of the controls. There was

EXPLANATION OF PLATE I
The 27-day Embryos
(Photomicrographs X330)

Fig. 3. Transverse section of the skin of an embryo from a control guinea pig. a, epidermal layer; b, subcutaneous tissue.

Fig. 4. Transverse section of the skin of an embryo from an experimental guinea pig. a, epidermal layer; b, subcutaneous tissue.

Fig. 5. Transverse section of the intestine of an embryo from a control guinea pig. a, epithelial lining; b, basement layer; c, lumen.

Fig. 6. Transverse section of the intestine of an embryo from an experimental guinea pig. a, epithelial lining; b, basement layer; c, lumen.

PLATE I

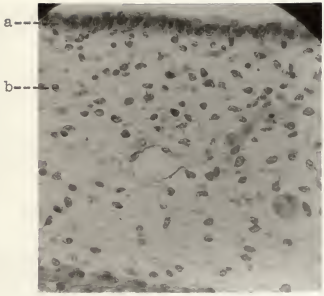

Fig. 3.

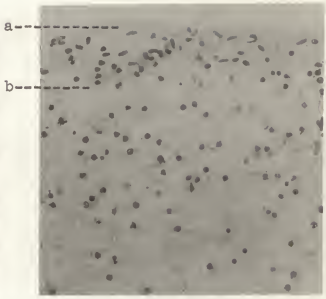

Fig. 4.

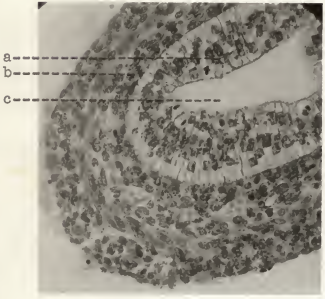

Fig. 5.

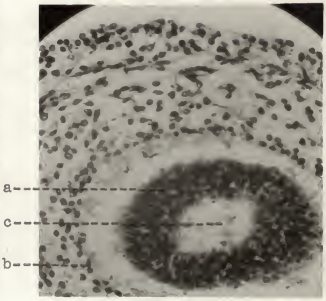

Fig. 6.

some indication of the digits but they were not as fully developed as were the digits of 27-day embryos of the control.

The difference in appearance of the developing follicles of the vibrissae was the same as that in the 27-day embryos.

A fold in the external ear in the fetuses of the control covered the external auditory meatus completely. Only a slight fold was present in the fetus of the experimental.

The folds which were forming the eyelids in the fetuses of the control partially covered the iris of the eye, while in the fetus of the experimental there was only a slight thickening which did not extend over the eyeball.

The internal structures of the fetus of the experimental were smaller and somewhat darker than the embryos of the control. The umbilical blood vessels were not distinct in the fetus of the experimental and they were harder to locate and appeared empty whereas in the fetuses of the control the blood vessels were distinct and filled with blood.

Similar contrast in the appearance and the rate of staining was noted between the tissues of the fetuses of the experimental and control animals as that in the tissues of the 27-day embryos, Plate II, Figs. 7, 8, 9, and 10.

The 34-day Fetus

The feet of the 34-day fetuses of the control were well developed with distinct nails and foot-pads. In the fetuses of

EXPLANATION OF PLATE II
The 29-day Fetus
(Photomicrographs X330)

Fig. 7. Transverse section of the skin of a fetus from a control guinea pig. a, epidermal layer; b, subcutaneous tissue.

Fig. 8. Transverse section of the skin of a fetus from an experimental guinea pig. a, epidermal layer; b, subcutaneous tissue.

Fig. 9. Transverse section of the intestine of a fetus from a control guinea pig. a, epithelial lining; b, basement layer; c, lumen.

Fig. 10. Transverse section of the intestine of a fetus from an experimental guinea pig. a, epithelial lining; b, basement layer; c, lumen.

PLATE II

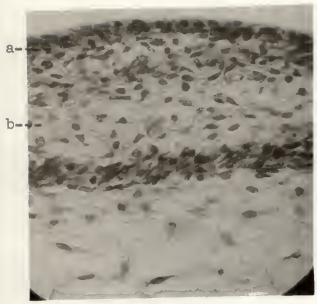

Fig. 7.

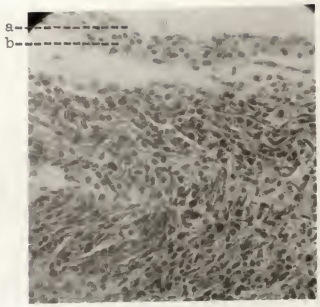

Fig. 8.

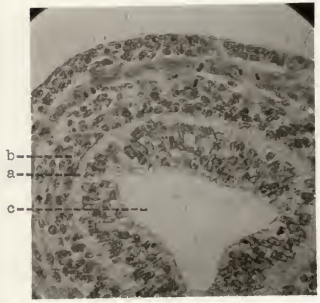

Fig. 9.

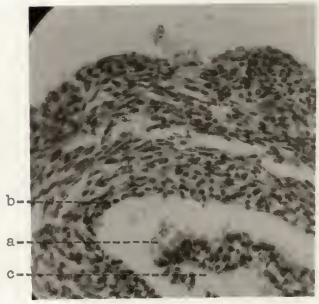

Fig. 10.

the experimental the toes were present but there were no nails or foot-pads.

The eyelids completely covered the eyes in the fetuses of the control but only about half covered the eye in the fetuses of the experimental.

The auricles of the ears flared back dorsally from the base in the fetuses of the control while in the fetuses of the experimental they folded ventrally over the external meatus similarly to that found in fetuses of the 29-day control.

Hair follicles appeared in considerable numbers posterior to the level of the eye in the fetuses of the control. No hair follicles appeared on the fetuses of the experimental.

In the fetuses of the experimental the internal structures appeared somewhat discolored. The blood vessels were difficult to follow and appeared to have no blood in them while the fetuses of the control had distinct blood vessels which were filled with blood.

The sexes were determined easily in the fetuses of the control but could not be determined in the fetuses of the experimental.

A small amount of dark red fluid was present in the uterus of the experimental guinea pig.

Plate III illustrates the contrast in the appearance of sections of tissues from fetuses at this stage. A thickening in the epidermal layer appeared for the first time in the fetuses of the experimental (Plate III, Fig. 12, a). The distribution

of the nuclei in the subcutaneous tissue appeared to indicate that cells do not occur as thickly here as they do in the same region of the controls (Fig. 12, b). The distribution is more nearly comparable to that seen in the sections of the 27-day embryos of the controls. Indistinct cell outlines were similar to that seen in previous stages.

Differentiation of muscular tissue has taken place in the intestine of the fetuses of the control (Plate III, Fig. 13, d) but no evidence of this differentiation was evident in the intestine of the fetuses of the experimental (Plate III, Fig. 14).

The 40-day Fetus

The 40-day fetuses of the controls appeared similar to the 34-day fetuses except that they were larger. Toe nails had lengthened into distinct claws. Some whiskers were present about the nose. Hair follicles were present over the head and back regions.

Two dead fetuses were secured at this stage of development from an experimental animal. Considerable resorption had occurred in one fetus and resorption to a lesser extent had occurred in the other. In these the eyelids did not cover the eye. The auricle of the ear lay in a small fold over the external meatus. The toes were present but there were no toe nails. These fetuses, in development, seemed to be at about the 27-29-day

EXPLANATION OF PLATE III
The 34-day Fetus
(Photomicrographs X330)

Fig. 11. Transverse section of the skin of a fetus from a control guinea pig. a, epidermal layer; b, subcutaneous tissue.

Fig. 12. Transverse section of the skin of a fetus from an experimental guinea pig. a, epidermal layer; b, subcutaneous tissue.

Fig. 13. Transverse section of the intestine of a fetus from a control guinea pig. a, epithelial lining; b, basement layer; c, lumen; d, muscle.

Fig. 14. Transverse section of the intestine of a fetus from an experimental guinea pig. a, epithelial lining; b, basement layer; c, lumen.

PLATE III

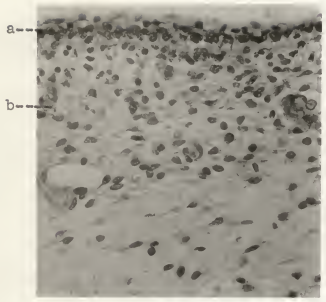

Fig. 11.

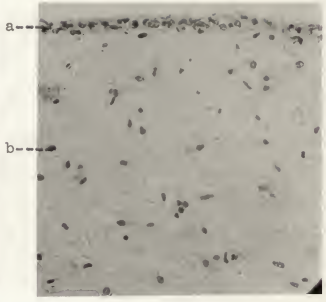

Fig. 12.

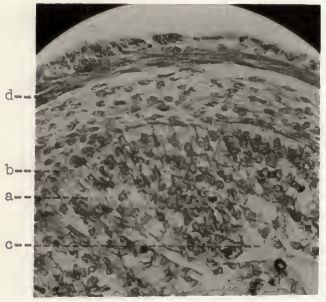

Fig. 13.

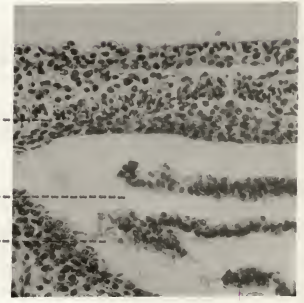

Fig. 14.

normal level but in weight and body length they were larger than the 34-day fetuses of the controls.

These embryos were aborted and a large amount of dark fluid was lost by the mother.

Plate IV shows the contrast in appearance of tissues of fetuses from control and experimental animals at this stage. The tissues of the fetuses of the experimental stained somewhat more readily than those described previously. The structure of the cells appeared better organized and more distinct. There was little differentiation of the epidermis in the fetuses of the experimental whereas this area is well differentiated in the fetuses of the control (Plate IV, Figs. 15, 16).

Hair follicles were beginning to differentiate in the skin of the fetuses from the control, but no hair follicles were present in the skin of the fetus from the experimental.

The muscle layer has appeared in the intestine of the fetus from the experimental (Plate IV, Fig. 18, d), but it has not developed beyond that seen in the intestine of the 34-day fetus from the control (Plate III, Fig. 13, d). The intestine of the fetus of the experimental (Plate IV, Fig. 18) presents a shrunken appearance with walls much thinner than the corresponding tissue from the fetus from the control (Plate IV, Fig. 17).

The 42-day Fetus

The 42-day fetuses of the control were similar to the 40-

EXPLANATION OF PLATE IV
The 40-day Fetus
(Photomicrographs X330)

Fig. 15. Transverse section of the skin of a fetus from a control guinea pig. a, epidermal layer; b, subcutaneous tissue.

Fig. 16. Transverse section of the skin of a fetus from an experimental guinea pig. a, epidermal layer; b, subcutaneous tissue.

Fig. 17. Transverse section of the intestine of a fetus from a control guinea pig. a, epithelial lining; b, basement layer; c, lumen; d, muscle.

Fig. 18. Transverse section of the intestine of a fetus from an experimental guinea pig. a, epithelial lining; b, basement layer; c, lumen; d, muscle.

PLATE IV

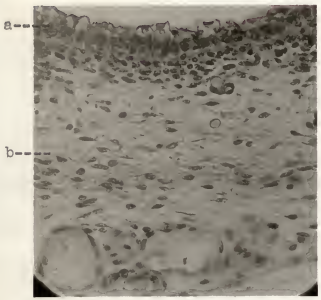

Fig. 15.

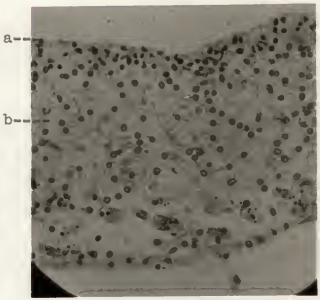

Fig. 16.

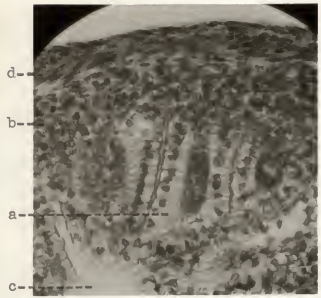

Fig. 17.

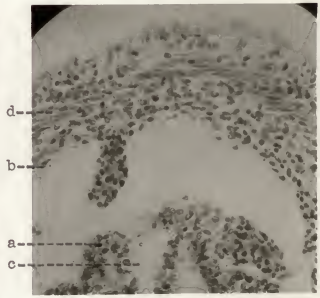

Fig. 18.

day stage with a slight increase in size.

The fetuses of the experimental were dead, but were not aborted. A large volume of very dark, nearly black, fluid was found in the uterus. One fetus was resorbed considerably; the eyelids did not cover the eyes; there were no toe nails, and the flexures were about the same as the 27-day stage. It weighed 1.1 g and was 2.0 cm in length. The other embryo weighed 3.75 g and was 5.0 cm in length. No hair follicles were present. Toe nails were differentiated. The auricles of the ear flared dorsally, and were larger than the 34-day normal ear flange, but not so large as in the 40-day fetuses of the controls.

No sections of the tissues were made at this stage.

The 50-day Fetus

The 50-day fetus was the oldest fetus studied. Two fetuses from experimental animals and five from controls were secured. The fetuses from the experimental were smaller than those from the control, Table 1. The two from the experimental were identical in length and weight, and similar in general appearance.

There was a dark red discharge from the vagina at the time the fetuses were removed and the mother appeared sick and had lost weight. The uterus was filled with a dark red fluid. The loss of blood in removing the fetuses from the experimental mother was small as compared to that lost by the control mother.

The placental discs, Table 2, and the umbilical veins were

smaller in the experimental than were those of the control.

The skin of the fetuses from the experimental was thin, easily broken, and was poorly attached to the subcutaneous tissue. The skin of the fetuses from the control was thicker, tougher, and firmly attached to the subcutaneous tissue.

The fetuses from the experimental animal had hair follicles on the head and back region. Whiskers measuring 2 mm to 3 mm in length were present about the nose. In fetuses from the control, hair follicles were found over the head, back, belly, and on legs. Hair was present on the head and back and the whiskers on the face measured from 1/2 cm to 1 cm in length.

The embryonic and maternal parts of the placental disc were more firmly attached in the controls than in the experimentals.

The external ear was much larger in the fetuses from the control, measuring on the average 1.2 cm in diameter while the experimentals averaged 0.74 cm in diameter.

The internal structures of the fetuses of the experimental animal were somewhat darker and smaller than the fetuses of the control. Blood vessels in general in the fetuses from the experimental animal were small, difficult to find and except for the large veins did not have blood in them. In the fetuses of the control, blood vessels were large, distinct, and filled with blood.

The bones of the fetuses of the control were firm and hard, while the bones of the fetuses of the experimental were soft and easily bent.

The hearts of the fetuses of the experimental animal were dark in color. Gross measurements of these hearts averaged 7 mm by 4.5 mm. The hearts of the fetuses of the control animal were lighter in color and measured an average of 12 mm by 9.5 mm.

The gall bladder was well defined in the fetuses of the control, but indistinct in the fetuses of the experimental.

The thyroid glands in the fetuses of the control were large, pale in color and sharply convoluted. In the fetuses of the experimental they were smaller, darker in color, and only slightly convoluted.

Hair follicles were well developed in the skin of the fetuses of both the experimental and control animals, Plate V, Figs. 19 and 20. Some breakdown of cell walls and blurring of cell outlines were present in the fetuses of the experimentals, but this was not as marked as in previous stages. The nuclei are closer together in the tissue from the fetus of the experimental animal. The walls of the intestine of the fetus from the experimental were thinner than they were for fetuses of the control. The closeness of the nuclei appears to be the result of a shrinking of the cytoplasm of the cell of the fetus of the experimental animal, Plate V, Fig. 22. The section of the intestine from the fetus of the control, Plate V, Fig. 21, shows sharp cell outlines, layers of muscle fibers, a steeply convoluted basement layer, and tall columnar cells of the epithelial lining. The section of intestine from the fetus of the experimental animal, Plate V, Fig. 22, shows blurred cell outlines, a continuous layer

EXPLANATION OF PLATE V
The 50-day Fetus
(Photomicrographs X330)

Fig. 19. Transverse section of the skin of a fetus from a control guinea pig. a, epidermal layer; b, subcutaneous tissue; c, hair follicle.

Fig. 20. Transverse section of the skin of a fetus from an experimental guinea pig. a, epidermal layer; b, subcutaneous tissue; c, hair follicle.

Fig. 21. Transverse section of the intestine of a fetus from a control guinea pig. a, epithelial lining; b, basement layer; c, muscle.

Fig. 22. Transverse section of the intestine of a fetus from an experimental guinea pig. a, epithelial lining; b, basement layer; c, muscle.

PLATE V

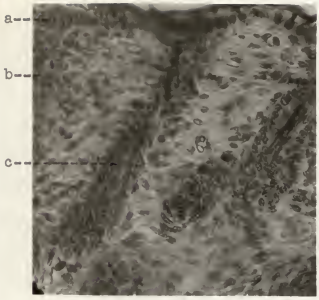

Fig. 19.

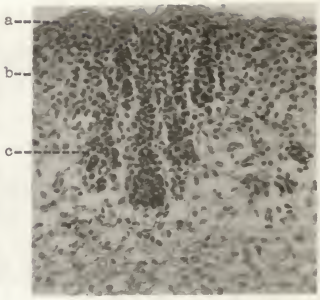

Fig. 20.

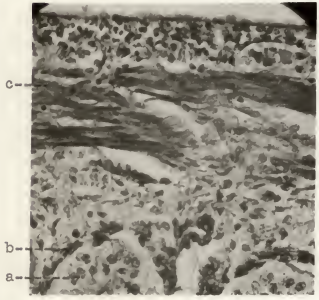

Fig. 21.

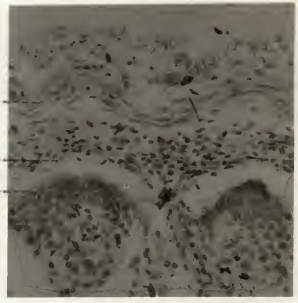

Fig. 22.

of muscle cells, a basement layer convoluted in waves, and sloughing of cells of the epithelial lining.

DISCUSSION

The appearance of the sections of tissues of fetuses from experimental animals at each stage and the fact that blood is found only in small quantities in the blood vessels of the fetuses indicate that there must be rather extensive loss of fluid from the fetus in vitamin C-deficiency in the mother. Harman and Kordisch (1945) observed circulatory changes which made it difficult to secure blood samples from the ear of vitamin C-deficient adult guinea pigs.

Death of embryos and abortion or resorption occurred in several cases such that the embryos could not be used for study, even though the mothers had not been placed on the vitamin C-free diet until the 21st day of gestation. This is in accord with the findings of Kramer, Harman, and Brill (1933) in which they observed abortion or resorption occurring in every case of pregnancy of vitamin C-deficient guinea pigs when the vitamin C-free diet was employed before the 29th day of gestation.

Indefinite cell boundaries and blurred appearance of tissues observed in the sections of tissues of fetuses from vitamin C-deficient animals were similar to conditions found by Kramer, Harman, and Brill (1933) in the stratum granulosum and the discus proligerous, and atresia of connective tissue in the ovaries of

vitamin C-deficient guinea pigs.

Hemorrhage into the uterine cavity occurred in every case of advanced vitamin C-deficiency. Examination of the placental discs of the experimental animals showed a smaller size and a weaker attachment to maternal tissues than was found in those of control animals. Tissue breakdown was probably present here. Maternal and embryonic parts of the discs could be pulled apart easily. Saffrey, Harman, and Kramer (1937) observed endometrial hyperplasia in pregnant vitamin C-deficient guinea pigs. Unusually large glands and the beginning disintegration were observed by them. Breakdown of tissue in either the area of the placental disc or endometrium or both, resulting in loss of blood, may be a factor in causing the observed retarded development and breakdown of tissues in the fetuses.

The reduction of cytoplasm which resulted in shrinking of tissues and nuclei appearing closer together is comparable to the findings of Harman and Traulsen (1943). In their study of connective tissue they found more nuclei per unit of area of sections of muscular tissue from vitamin C-deficient adult guinea pigs than were found in tissues from controls.

CONCLUSIONS

Vitamin C-deficiency in the mother guinea pig results in very marked effects upon the embryos she is bearing. The effects observed were:

1. Embryos from experimental animals were smaller than embryos of corresponding copulation age from control animals.

2. Embryos from experimental animals showed a retardation of development.

3. Thickening of the epidermal layer in the skin occurred before the 27-day stage in embryos of the control animals, but was not seen in embryos of experimental animals until the 34-day stage.

4. Muscular tissue was differentiated in the 34-day stage of the fetuses from the control but was not observed until the 40-day stage in the fetuses from the experimental.

5. The cytoplasm of the cells in the intestine of embryos from experimental animals was reduced. The walls of the intestine were thinner and the nuclei were closer together than found in the intestine of embryos of control animals.

6. The epithelial lining was sloughed off in the intestine of the embryos from experimental animals.

7. Tissues from embryos of experimental animals did not take the cytoplasmic stain as readily as did tissues from embryos of control animals.

8. Cell walls in embryos of experimental animals were indistinct and blurred in appearance while cell walls were well defined in corresponding tissues of embryos from control animals.

9. Internal structures were smaller in embryos of experimental animals than corresponding structure of embryos of the same age from control animals.

10. The skin of embryos of experimental animals was thin, easily broken, and weakly attached to subcutaneous tissue while in embryos from control animals it was in each case thicker, tougher and securely attached to the subcutaneous tissue.

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