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*I hereby recommend that the thesis prepared under my supervision by* HENRY B. HALE

*entitled* FUNCTIONAL AND MORPHOLOGICAL ALTERATIONS OF THE REPRODUCTIVE SYSTEM OF THE FEMALE RAT FOLLOWING PREPUBERAL TREATMENT WITH ESTROGENS

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*Approved by:* Charles L. Leichert

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# FUNCTIONAL AND MORPHOLOGICAL ALTERATIONS OF THE REPRODUCTIVE SYSTEM OF THE FEMALE RAT FOLLOWING PREPUBERAL TREATMENT WITH ESTROGENS

A dissertation submitted to the

Graduate School of Arts and Sciences
of the University of Cincinnati

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by

Henry Bixby Hale

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TABLE OF CONTENTS

# FUNCTIONAL AND MORPHOLOGICAL ALTERATIONS OF THE REPRODUCTIVE SYSTEM OF THE FEMALE RAT FOLLOWING PREPUBERAL TREATMENT WITH ESTROGENS

Two years ago investigators in this laboratory (Weichert and Kerrigan, 1942) reported suppression of uterine gland, Fallopian tube, and ovarian development accompanied by intense cornification of the vaginal epithelium, hyperplasia of the myometrium, and an atypical pseudo-stratified uterine epithelium in 14-day-old female rats treated with estrogens from birth. These effects were the result of daily injections of estrone or diethylstilbestrol into the mother rats. The present investigation is concerned with the after-effects either immediately following such premature stimulation or in later life.

At the time the work on this problem was started, only a few reports of studies of a similar nature had appeared in the literature. Greene and Burrill (1941), in a brief account, stated that female rats given single large doses of estradiol dipropionate at birth, when examined 3-18 months later, had markedly enlarged oviducts showing inflammatory changes, small ovaries lacking corpora lutea, and uteri showing squamous metaplasia. These animals had remained in estrus persistently. In another brief statement, Turner (1941) pointed out that newborn female rats, given 100 to 200 I.U. of estrogen daily for the first ten days of life, later showed prolonged estrous periods, occasionally copulated without impregnation, and at ten months of age had ovaries which contained follicles undergoing atresia and lacking corpora lutea. More recently, Wilson (1943) treated young female rats with estradiol dipropionate for 28-day periods with total doses ranging between 0.037 mg. and 2.4 mg. The effects

observed later in life indicated that treatment begun at birth or prior to the 15th day permanently altered sexual behavior. Treatment started on the 15th day or later did not have this effect. The most severe modifications resulted from treatment begun at birth. The following effects were noted: (1) complete lack of spontaneous sexual behavior and inability to respond to estrus-inducing hormones given in amounts sufficient to cause estrus in normal rats; (2) small ovaries lacking corpora lutea and containing small atretic follicles along with atrophic interstitial tissue; (3) small uteri which showed several pathologic conditions and responded poorly to estrogenic stimulation; and (4) abnormally thickened mammary ducts showing irregular and profuse branching.

The present report presents an account of the after-effects in female rats treated with diethylstilbestrol or with estrone in the manner described by Weichert and Kerrigan (1942), in which estrogens were administered to lactating mothers. In addition, observations are included on a large group of rats treated with diethylstilbestrol at early ages by other methods of administration.

## MATERIALS AND METHODS

A total of 158 female albino rats of an inbred colony of the Sprague-Dawley strain was used. On the basis of methods of administration, they are grouped as follows:

(1) Ten animals from 7 litters were treated for the first 14 days of life by giving the mothers daily injections of 0.1 mg. diethylstilbestrol or 0.5 mg. estrone. At the end of treatment they were placed with foster mothers and reared to maturity. Ten additional females

from 3 other litters were treated similarly from day 7.

(2) Eighteen females from 9 different litters were given from 1 to 14 daily injections of 0.01 mg. diethylstilbestrol beginning on the 7th, 14th, 21st, or 28th days. Since the vaginas of 61 untreated littermates opened prematurely merely as a result of their being with the injected animals, they were retained for study along with 59 other animals whose vaginas had also opened prematurely. The latter animals had been in cages in which the nesting material had been sprayed with a solution of diethylstilbestrol and ether (0.1 mg. diethylstilbestrol in 20 cc. of ether) when the animals were either 2 or 8 days old. In some cases the cages had been treated only once; others had been sprayed as many as 5 times in a period of 10 days. The cages had not been cleaned for 21 days or longer.

Daily vaginal smears were prepared from the time that the vaginas opened. At maturity many were caged with vigorous males whose fertility was afterward tested with normal females. A few attempts were made to determine the reactions in the adult state to estrogens and to a gonadotropic preparation (antuitrin-S). Unilateral ovariectomy was performed in several cases in an effort to alter vaginal sequences. Material for histological study was taken from representatives at ages ranging between 14 and 460 days. Twenty-one normal animals were used as controls.

## RESULTS

Time of vaginal patency. The vaginal response occurred after 9-10 days in animals receiving estrone from mothers injected from the day of parturition. The time was slightly longer (10-13 days) among those

groups given diethylstilbestrol by direct injection, injections into lactating mothers, and injections into littermates. In females receiving diethylstilbestrol from contaminated shavings, the time varied in inverse proportion to the number of sprayings; a single spraying induced the response after 19-20 days, but 5 sprayings reduced the time to 11 days. The average age for the establishment of the vaginal introitus in 21 normal control females was 49 days.

Estrous cycles. As determined by daily vaginal smears, the ages at which estrous cycles first appeared in the experimental animals were quite similar to the ages at which puberty occurred in the control animals. In some cases, cycles were aberrant from the very beginning; whereas in others, cycles were normal during the first part of life but became abnormal at advanced ages. The age at which this change occurred varied between 4 and 10 months. Characteristically, the experimental animals were found to exhibit persistent vaginal estrus. Some had recurring periods of prolonged estrus, usually without a proestrous stage, but many remained in vaginal estrus for very extended periods. One outstanding case is that of an animal for which no diestrous smears were recorded during the entire life span of 381 days. Only 2 cases out of 84 were of the anestrous type; but even in these there were persistent-estrous intervals on rare occasions. Animals treated at earlier ages were found to be more greatly affected. (table 1) Smears often contained thick masses of cornified cells and large numbers of leucocytes. This type of smear was often misinterpreted and recorded as metestrus. However, histological studies showed that vaginas in animals exhibiting this type of smear were cornified and that the presence of leucocytes was due to inflammations in the upper part of the reproductive tract. Smear records for 21 control animals

showed no tendency toward persistent estrus. Smears were taken from the time of puberty until they were approximately a year old.

TABLE I

TIME OF TREATMENT WITH ESTROGENS IN RELATION TO ESTROUS BEHAVIOR

| AGE WHEN GIVEN INITIAL TREATMENT | TYPE OF ESTROUS BEHAVIOR | | |
|---|---|---|---|
| | CYCLIC | PERSISTENTLY ESTROUS | ANESTROUS |
| (days) | (no. animals) | (no. animals) | (no. animals) |
| 1-2 | 4* | 55 | 2 |
| 7-8 | 4 | 3 | 0 |
| 13-14 | 3 | 3 | 0 |
| 21-22 | 7 | 0 | 0 |
| 28 | 3 | 0 | 0 |

*Received lowest dosage; vaginas opened 19-20 days after beginning of treatment.

Mating behavior and reproduction. The majority of the animals of the group given treatment as early as the first or second days of life copulated when tested at mature ages, but only one out of 36 became pregnant and delivered young. This animal was one of a litter treated by spraying the shavings with diethylstilbestrol; the dosage was the lowest used, and vaginas in these animals had opened only after a lapse of 20 days. Only two of her young were found; both survived and were given good care by the mother. Although cycles were normal both before and after this gestation, the animal never became pregnant again. Only a few cases of pseudopregnancy were noted. All animals of the group given treatment for the first time at 21 days of age had normal cycles, copulated, and bore litters. Two females later failed to become pregnant, and autopsies showed bilateral pyosalpinx in both cases. Two animals were observed to exhibit masculine copulatory behavior, but it was observed on only one occasion in each case.

Reactions to hormonal preparations. Anestrous animals, when treated for 15 days with diethylstilbestrol (either 0.01 mg. or 0.1 mg. per day), exhibited fully cornified smears throughout the test period. Other animals with a tendency toward persistent estrus, when treated during a diestrous period, showed the same reaction. The same effect was elicited when estrone was used. In contrast, normal control animals of the same age exhibited estrus only during the first part of the test period. Daily injections of 100 R.U. of PU (antuitrin-S) into a constant-estrous animal produced diestrous smears in 3 days. After 10 days of treatment, histological preparations showed a mucified condition of the vaginal epithelium. Large tubo-ovarian abscesses were found at autopsy. Although histological sections were made, no ovarian tissue could be found.

Effect of unilateral ovariectomy in persistent-estrous animals. Two animals were unilaterally ovariectomized when in estrus. Diestrous smears appeared on the following day. Cornified smears reappeared in only one case but only after 30 days. Unilaterally ovariectomized control animals exhibited normal vaginal sequences without interruption throughout the post-operative period.

Morphological changes. Out of a group of 15 animals in which treatment had been started on the 14th day of life or earlier, 10 showed abnormal conditions in the periovarial bursae, ovaries, or oviducts at the end of 14 days' treatment. Histological preparations showed that the ovaries were only slightly retarded, but that they were compressed and displaced due to certain conditions present in the periovarial space. The bursae in 3 of the animals were distended with blood. The oviducts showed no abnormalities and contained very little

blood. In 2 other cases conditions were similar, but the hemorrhage had occurred earlier, and polymorphonuclear leucocytes had invaded the hemorrhagic area, the ovarian stroma, the epithelium of the Fallopian tube, and were also present in scattered clumps in the lumen of the tube.

More extreme conditions were found in the 5 remaining cases. Here the bursae were abscessed, and the Fallopian tubes were distended with pus. Inflammatory conditions were especially severe in the epithelium and muscular layers of the tubes. The abscess in the ovarian bursa was walled off by fibrous tissue except in the region of the ostium; the ovaries were displaced and compressed, but they were not involved in the inflammation. Conditions in the uteri, cervices, and vaginas indicated a high estrogenic stimulation, but there was no inflammation.

Although the five other animals of the same age were examined after their vaginas had opened prematurely, no abnormalities were found. The uterine, cervical, and vaginal tissues had been stimulated only slightly. Similarly-treated littermates of this latter number were found to be normal in all respects at puberty and at later ages.

Findings in 71 animals treated for the first time within the first two weeks of life and reared to adulthood were also extreme. The incidence of pyosalpinx and chronic salpingitis was high in animals examined at ages ranging from 120 to 460 days. These findings are summarized in table 2. In many, other pathologic conditions, including pyometra, endometritis, cystic glandular hyperplasia, and squamous metaplasia of the epithelium of both uterus and Fallopian tube, were found frequently although varying in degree.

Inflammatory conditions in the oviducts varied greatly. In some,

only a moderate swelling of the cephalic part of the tube could be seen; whereas in others, the entire tube was greatly enlarged. In its most extreme form, adhesions developed which produced cysts of varying size. One of the largest weighed slightly more than 13 gm. and measured 48 mm. through its longest diameter. (Plate I) Adhesions frequently connected these cysts to other viscera or to the body wall. Where the latter condition occurred, it was difficult to free the cyst without removing adjacent tissue. In one animal a necrotic condition appeared over the site of the abscess; later this area became ulcerated. (Plate II) When these extremely large cysts were first found, their character was not understood. It was not until many other specimens were available that it was possible to see the stages representing their development. Sections through the larger ones often failed to include ovarian tissue. In one animal, when a large cyst became palpable, it and the uterine horn from the same side were removed under aseptic conditions and placed in separate tubes of nutrient broth. B. pyocyaneus was obtained in pure culture from both.

Ovaries from 38 animals of various ages ranging between 42 and 460 days were studied histologically. In general, they contained a few developing follicles, wide areas of interstitial tissue, and large vesicular follicles. Corpora lutea were not present except in those whose cycles had been normal during the first part of life and had become aberrant after 4-10 months of age. The corpora lutea found in these cases were old, regressing ones. The large follicles were either normal in appearance and contained ova or were merely large cysts filled with liquid. The longest diameter usually measured no more than .9 mm., but three cases stand out because of their extreme sizes, which were 16.8 mm.,

5.4 mm., and 3.7 mm.

Only 2 abnormal cases were found among 43 animals treated for the first time at 21 days or later. Corpora lutea were present in the ovaries of the two abnormal animals, and there had been no tendency toward persistent estrus. In one animal, the only abnormality was pyosalpinx; in the other, pyosalpinx was present along with an enormous cystic follicle. (Plate II, Fig. 5) One of these animals had one pregnancy.

It was possible to establish a figure for the natural incidence of tubal inflammations in this colony of rats since a large number of preserved specimens of reproductive tracts from mature females were available. Only 20 cases were found in 799 animals. None of these was of the nature of a tubo-ovarian cyst.

TABLE II

OCCURRENCE OF TUBAL INFLAMMATIONS AFTER ESTROGEN ADMINISTRATION

| AGE WHEN GIVEN INITIAL TREATMENT (days) | METHOD OF ADMINISTRATION | ANIMALS WITH INFLAMMATION | UNAFFECTED ANIMALS |
|---|---|---|---|
| 1-2 | from lactating ♀ | 9 | 0 |
| | from littermates | 2 | 3 |
| | from shavings | 39 | 12 |
| 7-8 | from lactating ♀ | 2 | 1 |
| | injected | 2 | 0 |
| | from littermates | 3 | 0 |
| | from shavings | 0 | 8 |
| 11-14 | injected | 1 | 2 |
| | from littermates | 3 | 14 |
| 21-22 | injected | 1 | 8 |
| | from littermates | 1 | 17 |
| 28 | injected | 0 | 2 |
| | from littermates | 0 | 14 |
| -- | untreated | 20 | 779 |

## DISCUSSION

The results obtained in this study indicate that early prepuberal treatment with diethylstilbestrol or estrone for short periods and with fairly low dosages produces the same lasting effects in the female rat as have been reported when other estrogenic preparations were used. (Greene and Burrill, 1941; Turner, 1941). Whereas the results in these experiments are different in some respects from those reported by Wilson (1943), who used estradiol dipropionate, they apparently differ only in degree. Prepuberal treatment started prior to the 15th day in both sets of experiments altered sexual behavior. In his animals, cycles were completely abolished, and the ovaries contained only small and atretic follicles and atrophic interstitial tissue; in the present experiments, persistent-estrus resulted, but the ovaries contained large cystic follicles and the interstitial tissue was not generally atrophic. Wilson concluded that prepuberal treatment with estrogen had so altered pituitary function that luteinizing hormone was not secreted. It is generally agreed among investigators who have concerned themselves with the problem of the persistent-estrous animal (Everett, 1939; Pfeiffer, 1939; Turner, 1941a and b; Bradbury, 1941; Greene and Burrill, 1941; Fels, 1941, 1942; and Pfeiffer, 1942) that this phenomenon is the result of lowered LH secretion. It therefore seems that the effect noted by Wilson (1943) is a more severe but not a different effect.

The response shown by adult animals in the present study during treatment with estrogens is unlike that of normal adults but is like that of castrated animals. (Morrell and Hart, 1941) The reaction to PU is in agreement with tests of Greene and Burrill (1941) and Pfeiffer (1942). The fact that unilateral ovariectomy brought on diestrus indicates that the amount of estrogen secreted in the persistent-estrous

animal is relatively small.

The high incidence of bursal and tubal inflammation in both young and mature animals is perhaps the most significant feature of the results. Such conditions are not uncommon in experimental animals (Everett, 1939: Fluhmann, 1939: Shay, Gershon-Cohen, Paschkis and Fels, 1939; Wilson, Young and Hamilton, 1940; Wilson, Hamilton and Young, 1941; Bradbury, 1941; Greene and Burrill, 1941; Turner, 1941a and b; Fels, 1941 and 1942; Greene, Burrill and Ivy, 1942; and Lacqueur, 1944). If a speculation may be permitted, it would seem that the treatment of infantile rats with estrogens causes periovarial hemorrhage which, in turn, leads to inflammatory conditions in the bursa and secondarily in the oviduct. Since estrogens induce premature vaginal patency, it is possible for bacteria to enter the reproductive tract. The inflammatory lesions remain during development and are seen in the adults as enormously enlarged tubal abscesses.

It seems significant that these effects may be induced in animals unintentionally administered small amounts of diethylstilbestrol at early ages. In fact, the results among such cases were often more extreme than in animals treated directly.

## CONCLUSIONS

Diethylstilbestrol and estrone administered to female rats at ages prior to the 15th day produced a persistent-estrous state after the animals reached maturity. Such animals were capable of copulating but failed to reproduce. Corpora lutea did not appear in the ovaries, but mature follicles, often large and cystic, were found. Extremely severe inflammatory conditions and certain atypical uterine conditions

appeared frequently. Results with these estrogenic preparations do not appear to be different from those induced with other preparations.

## SUMMARY

The administration of diethylstilbestrol or estrone to female rats beginning either at birth or as late as the 14th day and for so short a time as 14 days produced permanent functional and morphological changes in the reproductive system. Typically, these animals tended to remain in vaginal estrus. Some had recurring periods of prolonged estrus, and many remained in vaginal estrus for such extended periods as 381 days. Cycles were usually aberrant from the time of puberty, but in a few cases, persistent estrus did not appear for 4 to 10 months. Persistent-estrous animals copulated frequently, but none became pregnant; whereas animals treated for the first time at 21 days had normal cycles, copulated, and bore litters. Vaginal estrus was induced readily and could be maintained without interruption when animals in diestrus were given diethylstilbestrol or estrone over a two weeks' period. Diestrous smears were induced in constant estrous animals by the administration of PU and by unilateral ovariectomy. Ovaries of adult animals given prepuberal treatment were generally small and contained a few developing follicles, numerous large vesicular follicles, and a considerable amount of interstitial tissue. A high incidence of bursal inflammation, pyometra, cystic glandular hyperplasia, and squamous metaplasia in the uterus and Fallopian tube was noted. Pyosalpinx or chronic salpingitis occurred in 61 per cent of the cases initially treated on or before the 14th day of life. Large tubo-ovarian cysts were found

frequently. One of these, and a portion of the uterus, removed aseptically, showed B. pyocyaneus in pure culture.

It is significant that these conditions appeared in many animals treated accidentally by being in contact with animals injected with diethylstilbestrol or in cages contaminated with this preparation.

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PLATE I

Fig. 1 Reproductive tract of mature animal showing tubo-ovarian cysts resulting from the administration of diethylstilbestrol to mother during the first 14 days following parturition.

Fig. 2 Specimen from animal unintentionally treated from day 7 to day 21. Injected littermates received 0.01 mg. diethylstilbestrol daily.

Fig. 3 Specimen from animal unintentionally treated from day 2 to day 28. Shavings in cage were contaminated with 0.5 mg. diethylstilbestrol.

Fig. 4 Specimen from animal unintentionally treated from day 2 to day 22. Shavings in cage were contaminated with 0.3 mg. diethylstilbestrol.

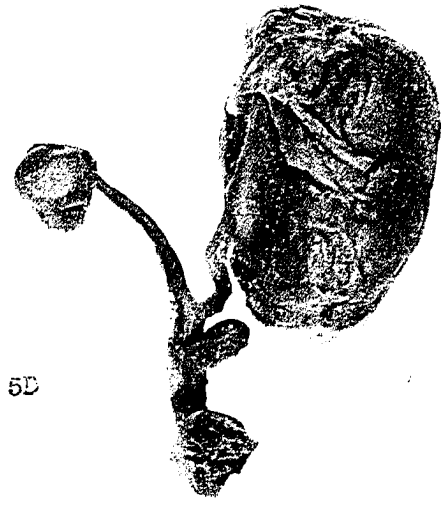


Fig. 1

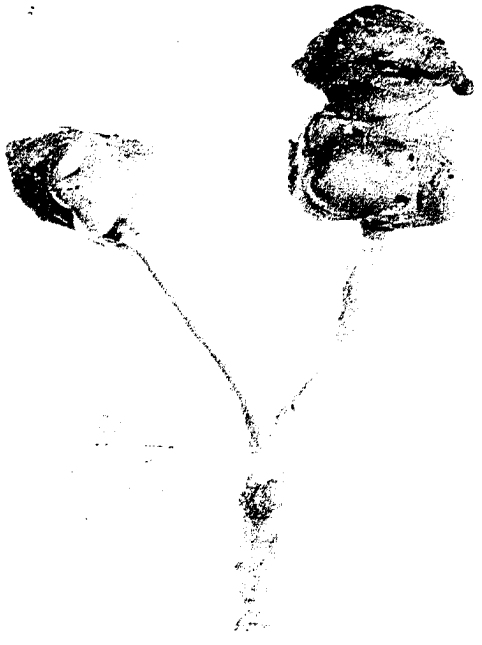

Fig. 2

Fig. 3

Fig. 4

PLATE II

Fig. 5 Specimen from animal unintentionally treated on day 22. Littermate received a single injection (0.01 mg. diethylstilbestrol). Right ovary contained a follicle measuring 16.8 mm. through its longest diameter. Note convolutions of enlarged oviduct on surface of cyst; adhesions were not present.

Fig. 6 Animal showing ulcerated area over site of tubal abscess. Remainder of reproductive tract is shown to the side. Animal was unintentionally treated from day 2 to day 27. Shavings in cage were contaminated with 0.3 mg. diethylstilbestrol.

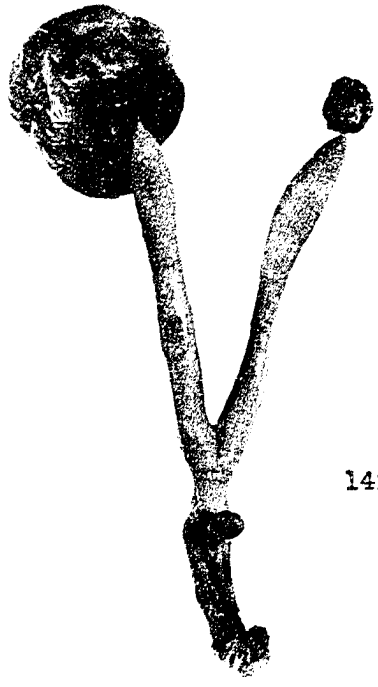

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Fig. 5

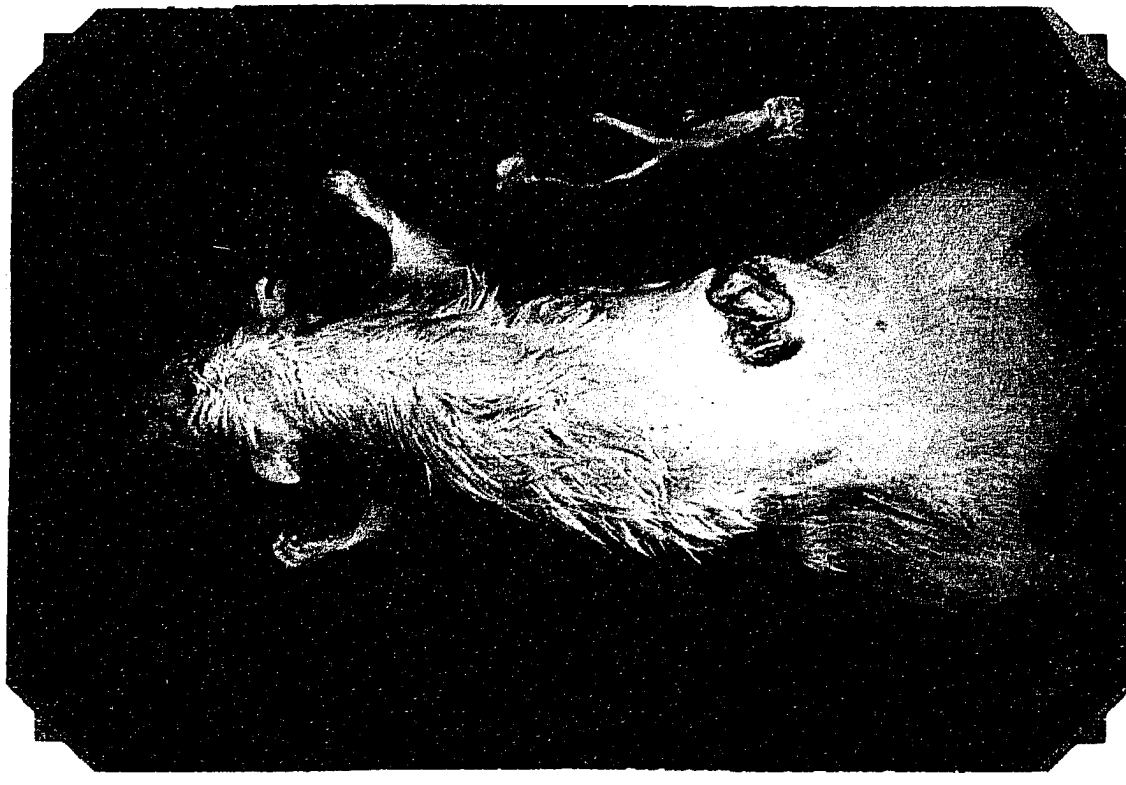

Fig. 6