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THE EFFECT OF HEPARIN ON GROWTH CHARACTERISTICS OF TRANSPLANTED SPONTANEOUSLY OCCURRING C_3H/HeJ MOUSE MAMMARY TUMOR

A Thesis

Presented to

the Faculty of the Graduate School

University of the Pacific

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Muhammed Saleh Abdullah Banaja

July 1981

DEDICATION

I dedicate this thesis

to

my parents, who showed me the way, and

to

dear God who made everything possible.

1982

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INTRODUCTION

Mouse mammary tumor is a common form of cancer used for experimental purposes. It is relatively resistant to chemotherapy. This investigation is designed to test the effect of heparin, a naturally occurring anticoagulant, on tumor growth using a genetically uniform laboratory animal.

The C_3H/HeJ Mouse

Dr. Clarence C. Little was among the first to recognize the usefulness of genetic uniformity in reducing the variables associated with experimental work (1). He pointed out that genetically similar populations allow investigators to check their own observations and allow other researchers to verify the results (1). Use of the C_3H strain of mouse in the present project eliminates histocompatibility problems.

The C_3H/HeJ mouse, which is agouti in color (2) is a strain of the C_3H mouse developed by careful inbreeding. The J indicates that it is a subline of C_3H/St, a strain developed by Strong (2,3). The C_3H mouse was, in turn, developed from the C family and was initially introduced around the year of 1918 (4-12).

Between the years of 1918 and 1920 (2,3,4-13), Strong

crossed a female Bagg albino mouse with a DAB* male to produce the inbred strain C_3H which was developed separately from other strains (2, 4-12). In 1930, Strong gave the C_3H mouse to Andervant. About 1938 -1941, Andervant passed the C_3H/HeJ mouse at F35 to Heston (the F35 refers to the inbreeding of the 35th generation of brothers and sisters). In 1947-1948 Heston gave the F48, which is the 48th breeding of brothers and sisters, to Jackson Laboratory and in 1980 this laboratory purchased the F150 generation from Jackson Laboratory for this research.

The C_3H strain has more than 10 major sublines (14). Most of these sublines have good reproductive capacity (14) and exhibit a very high incidence of spontaneous mouse mammary tumor (2,3,13-16).

Strong and Heston have indicated that the incidence of the spontaneous developing mouse mammary tumor in the C_3H/HeJ mouse is highest in the breeding female (65%), lower in the virgin female (28%) and lowest in the male (2-3,17).

The C_3H/HeJ subline exhibits a uniformity in a variety of characteristics useful for experimental biology (1,2,13). Certain characteristics, such as coat color and rd (retinal degeneration gene), are highly uniform (2,4-11). Minor factors, such as the life span, susceptibility to constitutional disease, and other physiological characteristics are not

---

*Little's mice were used in color experiments and represent one of the oldest inbred strain (since 1907).

absolutely uniform because their expression is a function of both heredity and environment (2,13,18). The uniform genetic characteristics of the C_3H mouse are of great importance in certain aspects of cancer. These findings make the unfostered sublines appropriate models for research into mammary tumor biology (13, 18-21).

The C_3H/HeJ subline exhibits a poor immune response to endotoxic lipopolysaccharide (14); this is thought to be due to a B lymphocyte deficit. One explanation for this abnormality of the C_3H/HeJ immune system would be an altered hematopoeitic system since both low red and white cells counts are observed in the live animal (4-11).

An understanding of mammary tumor morphology requires study of normal and abnormal mammary tissue. In the following section, these topics will be discussed: (i) the normal mouse mammary tissue, including gross morphology and microscopic anatomy, and (ii) tumorous mouse mammary tissue, including pathogenesis and factors influencing tumorogenesis.

Normal Mouse Mammary Tissue

A C_3H/HeJ mouse has five pairs of mammary glands, three thoracic and two inguino-abdominal (22). Dissection of these glands shows that the healthy tissue is opaque, white in color and pulpy in texture (2, 23, 24).

Histologically, normal mouse mammary tissue undergoes various changes during puberty, pregnancy, lactation, and involution (20,25-28). The inactive mammary gland (Figure 1)

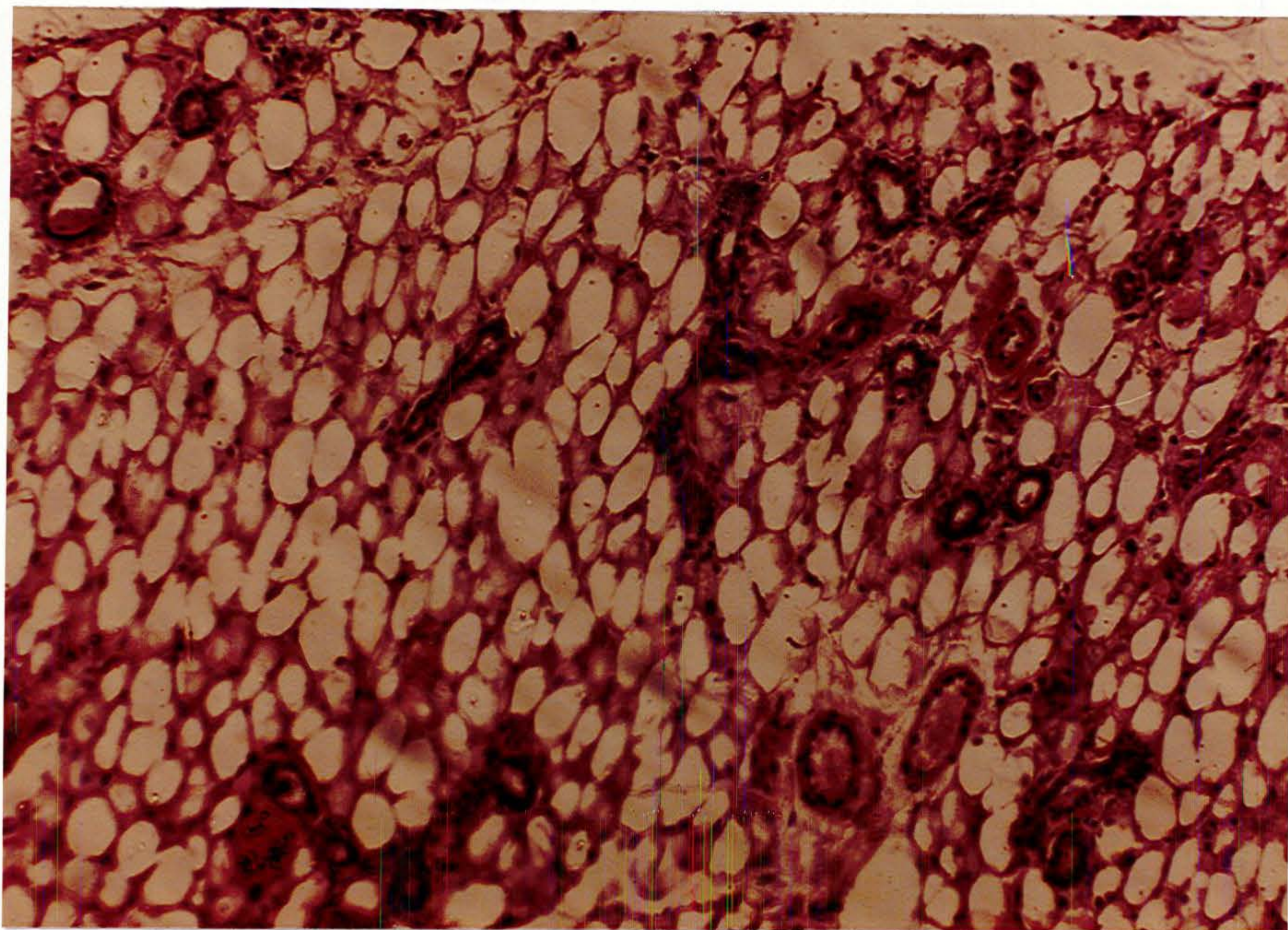

Figure 1. C3H/HeJ mouse inactive mammary tissue (10 X). Note the low ratio of glandular tissue (elements) to adipose tissue.

is hypoplastic with very few glandular elements (25). Pregnancy (Figure 2) induces remarkable growth of tubules and secretory alveoli (29). The alveoli are empty of secretions early in pregnancy (29, 30).

The most remarkable change occurs during lactation (Figure 3), when the alveoli increase in size. At this time, most of the alveolar lumens contain large volumes of lipid and protein secretions (31). This situation continues until the involutional stage (Figure 4). At that time, contraction of the glandular tissue takes place and the alveoli become smaller although they remain irregular (25,26).

Mouse Mammary Tumor

On April 24, 1946, a spontaneous mammary adenocarcinoma was detected in a nine-month-old female mouse of the strain C_3H. The tumor was characterized and transplanted (32). Since that time, mammary adenocarcinoma tissue has been transplanted successfully by subcutaneous, intramuscular, intraocular, intrahepatic and intraperitoneal inoculation.

Mouse mammary tumor is of two histological types: one consisting of small, uniform acini supported by minimal stroma; the other, a solid mass of tumor cells arranged in nodules surrounded by connective tissue (23,24,33). The latter (Figure 5) has been observed more often in the C_3H mouse (23,24,34).

Although the transplantability characteristic of

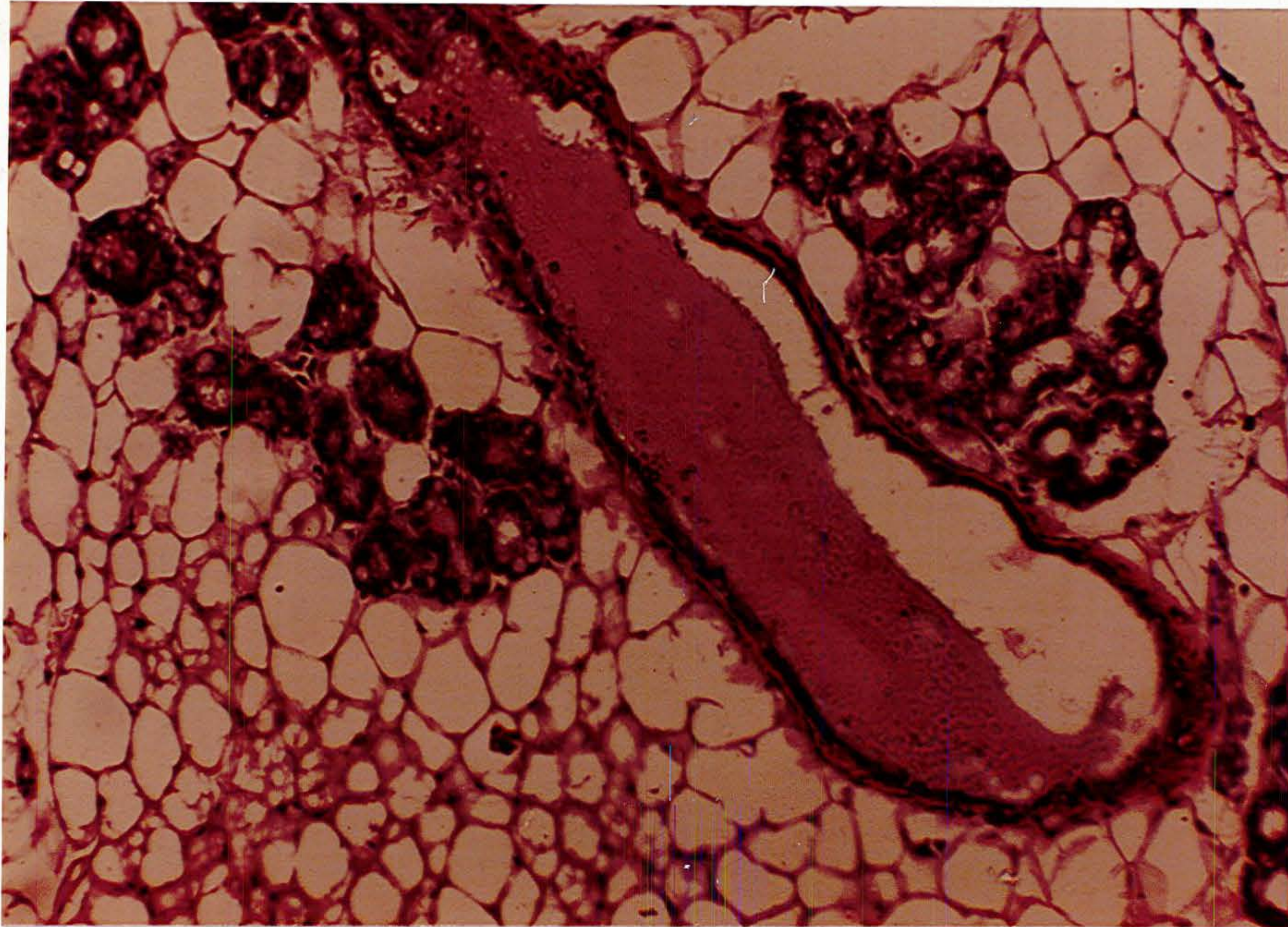

Figure 2. C_3H/HeJ mouse pregnant mammary tissue (10 X). Note the increase in relative numbers of mammary acini to adipose tissue. Also observable is the presence of lipid and protein secretions in ducts.

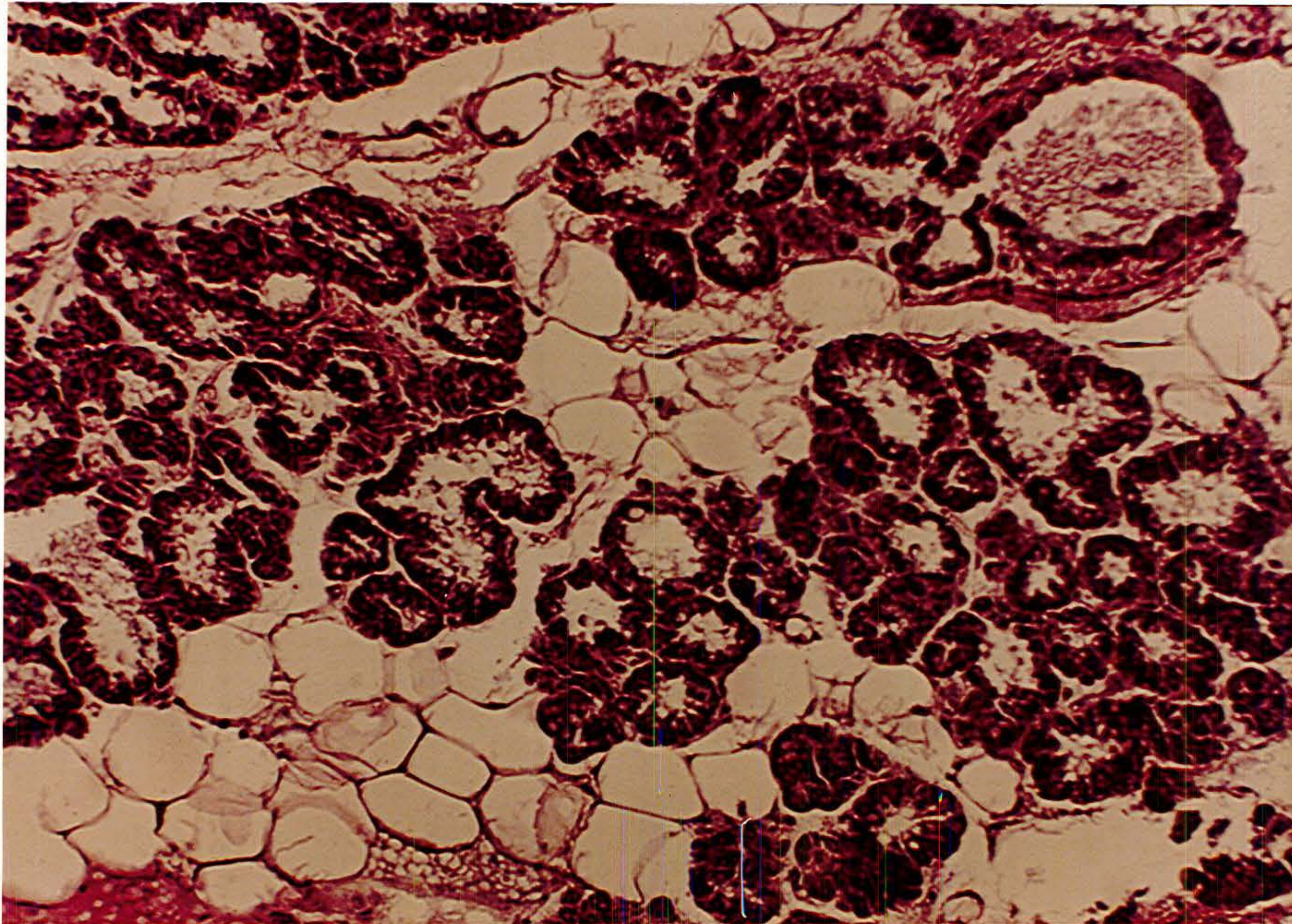

Figure 3. C_3H/HeJ mouse lactating mammary tissue (10 X). A striking increase in glandular elements with relative reduction in connective/adipose tissue can be observed.

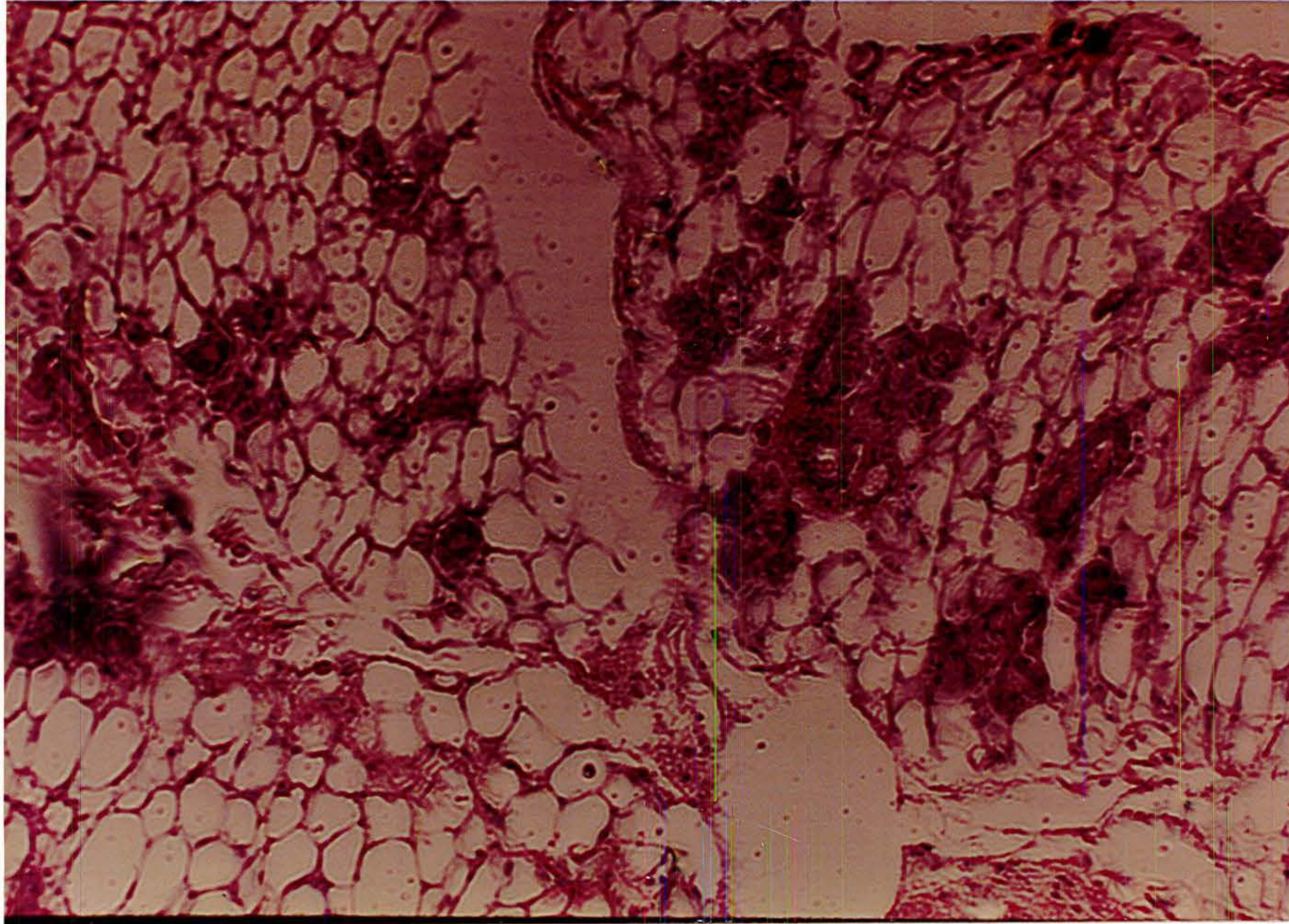

Figure 4. C3H/HeJ mouse involuting mammary tissue (10 X). Note a reduction of glandular elements; appearance is similar to the inactive state.

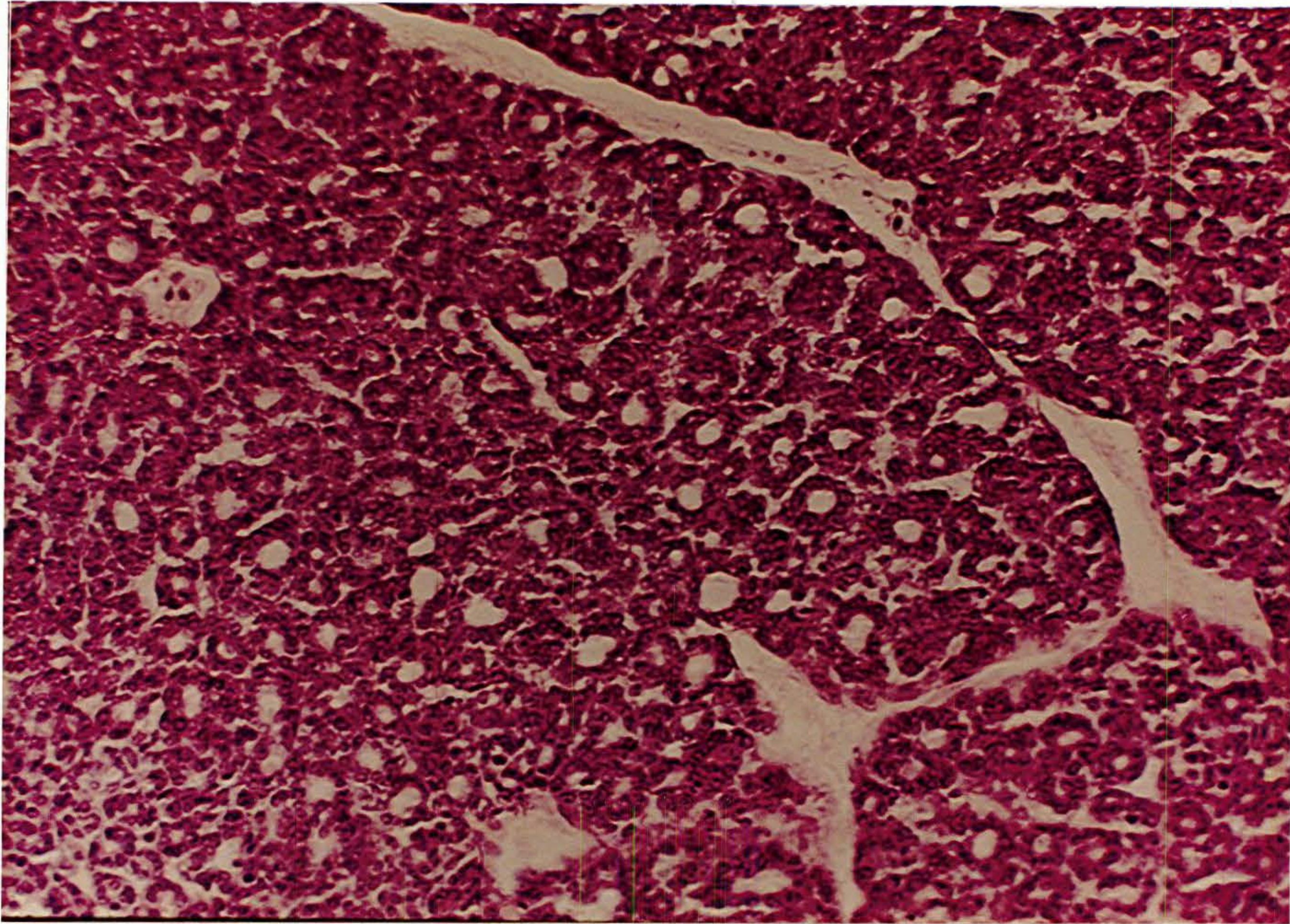

Figure 5. C3H/HeJ mouse spontaneous mammary tissue (10 X). A solid mass of glandular like tissue with scant connective tissue is shown here.

the mouse mammary tumor tissue is by far the most important indicator for its malignant nature (35), other characteristics such as anaplasia and rapid growth with demise (death) of the animal are also strong evidence for its malignant nature (15, 18).

Mouse mammary tumor virus has been known to be oncogenic since 1918. An RNA oncogenic virus, mouse mammary tumor virus (MMTV), is transmitted from an infected mother to the offspring by nursing (13, 15, 16).

The mouse mammary tumor virus can be identified in the milk (15, 16). A spontaneous mouse mammary tumor will develop in a genetically susceptible animal under the influence of this virus and probably other enviornmental factors (15). Several factors influence tumorogenesis (36) including:

(i) Virus: Other viruses in addition to mouse mammary tumor virus are known to cause mouse mammary tumor (15, 36, 37).

(ii) Genotype: The susceptibility of mice to develop spontaneous mouse mammary tumor is dependent on heredity (15, 16). In some resistant strains such as C57BL, I, 020 and C57BL/10, mouse mammary tumor virus will fail to develop a high mammary cancer incidence (15).

(iii) Hormonal Environment: Virus-induced mammary tumors are essentially hormone-independent

(16); they can grow not only in normal females but also in castrated females or in males (16). However, in susceptible (virus-carrying) strains of mice, such as the C_3H/HeJ mouse, hormonal stimulation results in much higher incidence of mouse mammary adenocarcinoma (16). This hormonal factor has been supported by many experiments showing that ovarian extracts, estrogen or estrogen-progestogen combinations do, in fact, stimulate oncogenesis (38); others such as glucocorticoids, dexamethasone, prolactin, and insulin also seem to play some role (39-45).

Tumor Transplantation

Tumor transplantation is a technique whereby a viable tumor can be transplanted from a donor animal to a syngeneic host. The value of this technique resides in the fact that tumor viability can be maintained continuously as cells are passaged from one animal to another. This technique is of great value in cancer and/or immunological research (46).

The history of tumor transplantation goes back more than a century when many investigators thought that the transplantable tumors might be better tools for cancer and/or immunological research than spontaneous or induced tumors. In 1876, M.A. Novinsky (1841-1914), known as the father of tumor transplantation, successfully transplanted, possibly

for the first time, a spontaneous malignant tumor. He used a dog because at that time small laboratory animals were not widely used (47).

Novinsky drew several conclusions: first, he noted that it was possible to perform implantation of a tumor; second, that inoculation success depended on several factors: (i) size of the initial incision of the skin, (ii) the size of the tumor inoculum, and (iii) the quantity of viable cells present in the transplant. He also suggested that tumor cells should be considered as infecting agents to healthy tissue, and that species identity between the donor and the host should be considered during inoculation (47).

The credit for successful tumor transplantation in laboratory animals has been misguidedly awarded to Hanau, who in November 28, 1888 successfully inoculated two rats with an epidermoid rat carcinoma. Hanau himself refers to Novinsky's work on tumor transplantation in dogs (47,48).

During the period of 1889-1952, Kanematsu Sugiura with three other authors, as well as four independent investigators, reported that rat and mouse tumors as well as venereal sarcoma in the dog could be successfully transplanted (47).

At the present time, there are more than 100 known transplantable tumors of the mouse, rat, hamster, guinea pig, rabbit, dog, and chicken.

Even minor factors (discussed later) play an important

role in the success of tumor transplantation. Successful transplantation of a solid tumor requires the following instruments: stainless steel dissecting scissors, forceps with medium point, curved dissecting forceps, dissecting knife and trocars (46). The trocar is a special stainless steel 13-gauge instrument made especially for tumor transplantation. The wide bore of the trocar is well known to cause less damage to the tumor cells.

In solid tumor transplantation, it has been recommended that the non-necrotic, nonhemorrhagic tumor mass be cut into small cubes prior to transplantation since the response in the host and tumor survival will depend on the size of the tumor mass (46).

Tumor transplant growth is facilitated if transplanted into a histocompatible host or an immunosuppressed host or an animal such as the nude mouse. Otherwise, the normal immune system, in particular T lymphocytes, may reject it (46, 47).

Because of the ease of transplantation, the most suitable site is into axillary subcutaneous tissue. It has been noted that tumor take and growth are best in young, healthy, vigorous mice and rats, regardless of sex although sexes should be separated to prevent pregnancy (46).

Successful mouse mammary tumor implementation can be detected at the implementation site within a week (46, 47). In the mammary adenocarcinoma transplant, rapid growth is generally observed, although this is dependent on several

factors such as inoculation size and site. Animal death occurs within weeks (46).

Other factors can contribute to the failure of transplanted tumor cells to establish themselves. These include: (i) inoculation of stationary or receding tumors, (ii) inoculation of stored frozen tumors, (iii) spontaneous regression of transplantable tumors, and (iv) resistance to inoculation (46,47).

Dvorak Model

In 1979, Dvorak *et al.*, postulated that solid tumors have the ability to stimulate the formation of a protective fibrin cocoon around the tumor (49). Dvorak suggested that this fibrin cocoon might enable the tumor cells to develop without challenge from the host's immune surveillance system or provide some other survival advantages (49-54).

Prior to cocoon formation, tumor cells release a vascular permeability factor (VPF) at an early stage after transplantation. This factor increases vascular permeability locally in the region of the tumor, allowing blood constituents, including fibrinogen, to escape into the tissue bordering the tumor. The VPF is not histamine mediated (49).

In addition to the release of the VPF factor, tumor cells release a procoagulation factor (PCF) which in turn triggers the blood clotting cascade leading to fibrin formation in the form of a cocoon. This has been observed to occur within hours after transplant (49).

In order to allow for future tumor growth, Dvorak postulated production of a third factor called plasminogen activator factor (PAF). PAF activates plasmin to stimulate fibrinolysis and as the tumor grows, the cocoon is then able to be reshaped.

A migration inhibitory factor (MIF), which has the capacity to inhibit migration of macrophages to the tumor zone, is postulated by Dvorak as an additional (fourth) factor promoting tumor growth (49).

The central core of the Dvorak hypothesis is the fibrin cocoon. He has suggested that the cocoon is advantageous for tumor growth since it may protect neoplastic cells from the host immune surveillance system. He also noted that the fibrin cocoon is apparently associated with the development of new blood vessels that penetrate the tumor (49). The presence of the fibrin cocoon has been confirmed by immunofluorescence as well as by electron microscopy.

Special Project

Other workers have noted a relationship between tumor growth and coagulation. In 1958, Wood demonstrated that tumor cells have to become engulfed in a fibrin clot as a prerequisite for metastasis (53). This was followed in 1960 by the work of Hiramoto *et al.*, who demonstrated the presence of extravascular fibrin deposits within tumors (54). In 1971 it became clear (Folkman) that a tumor fibrin matrix supporting a capillary network was essential

for tumor survival (55). Lastly, several investigators between the years of 1973-1981 (Elias et al.; Brown; Zacharski et al.; Poggi et al.), have shown that anticoagulants will protect the host against metastasis (56-60).

The present research project was designed to examine the effect of a naturally occurring anticoagulant (heparin) on tumor growth in an experimental animal. The hypothesis was to test the relationship between heparin and Dvorak's hypothesis using transplanted mammary tumors and the C_3H/HeJ female mouse, known for its high incidence of spontaneous mammary tumor. As a solid tumor, mammay tumor tissue is ideal for such investigation since solid tumors can be retrieved and weighed easily.

Heparin, since its discovery in 1916, has been used world wide clinically for its anticoagulation properties (61-80). The mechanism of action involves its highly charged acidic properties. Heparin action sites in the coagulation cascade are indicated in Figure 6 (66, 79). Although it has several sites of action, its dominant effect is the inhibition of thrombin formation.

Brambel proposed that heparin, with its cofactor antithrombin III, inactivates factor V by forming a "heparin cofactor-factor V heparin complex" (80). This results in a deficiency of the prothrombin activator complex which is normally composed of factor Xa (activated factor X) and

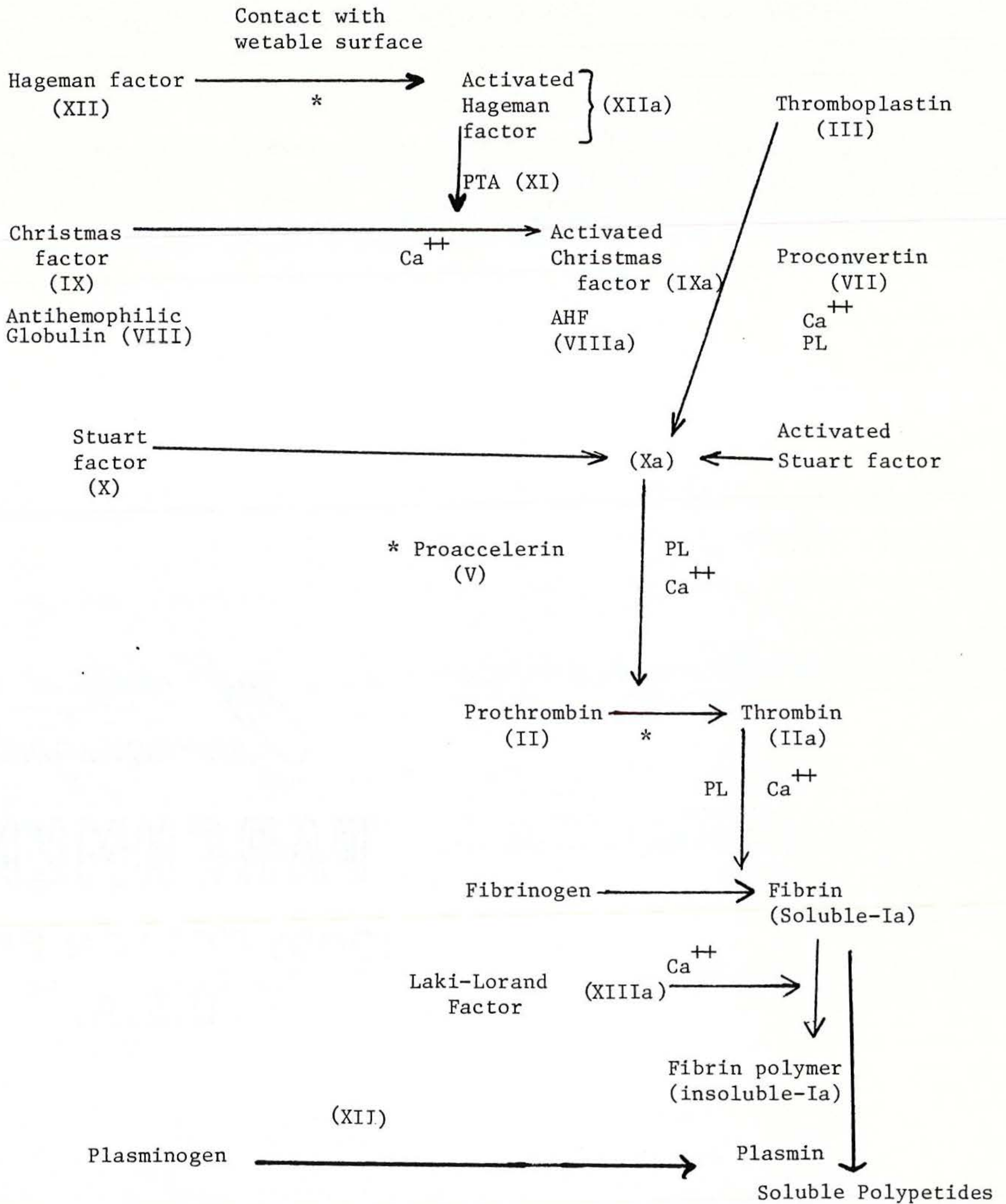

Figure 6. Intrinsic clotting sequence. (Known sites of heparin action are indicated with an asterisk.)

Factor V plus phospholipids and calcium. The net result of this inhibition is a decrease in thrombin production (66, 69, 79). The "heparin cofactor-factor V heparin complex" undoubtedly occurs because heparin is a strongly charged (negative) molecule (69).

Another observation has been made by Soulier and Lewis, who noted that the presence of a heparin cofactor, a plasma alpha-globulin called antithrombin III is essential (79,81,82) for its antithrombotic activity.

In the body, heparin is stored naturally in mast cells bound to histamine and 10-24 units %, (0.1 - 0.24 mg/ 100 ml) can be found circulating in blood plasma, where it presumably acts as a "natural" anticoagulant (man, horse) (69). In addition to its anticoagulant activity, heparin has some "antiinflammatory" properties. Dragstedt, in 1942, observed that histamine reacts with heparin to form a non-reactive (histamine-heparinate) complex, thereby inhibiting the release of histamine during an antigen-antibody reaction (83). This antiinflammatory action is not related to anticoagulant activity (84).

Heparin is also known to have some antilipemic and anticomplementary activity (67, 84).

MATERIALS AND METHODS

Tumor Transplantation

To obtain material for implantation, a female donor C_3H/HeJ mouse with a spontaneous mammary tumor (Figure 7) was sacrificed using diethyl ether. The dorsal surface of the dead animal was swabbed with 70% ethanol, an incision made, skin retracted and pinned back, and the tumor carefully dissected from the surrounding tissue. When separated from surrounding tissue, the tumor appeared as an opaque, yellowish-white mass. After several washings using sterile Ringer's solution (46), the tumor was dissected into cubes measuring approximately 2 x 2 x 2 mm (see Figure 8) with an average weight of 0.0179 g (observed range = 0.0115 - 0.0244 g).

Fifty-four (27 control), C_3H/HeJ females weighing 20-26 g were used as the host animals (85,86). Tumor implants of the above dimension and consisting of approximately 6.3×10^8 cells were used. Animals were exposed to diethyl ether for approximately 50 seconds until motor coordination was lost. With the animal prone, a 3 mm incision was made on the lower back after bathing the area with 70% ethanol (Figure 9). A 13-gauge needle, containing tumor tissue, was introduced subcutaneously into the tissue via the skin incision

Figure 7. C_3H/HeJ (donor) mouse with a spontaneous mammary tumor.

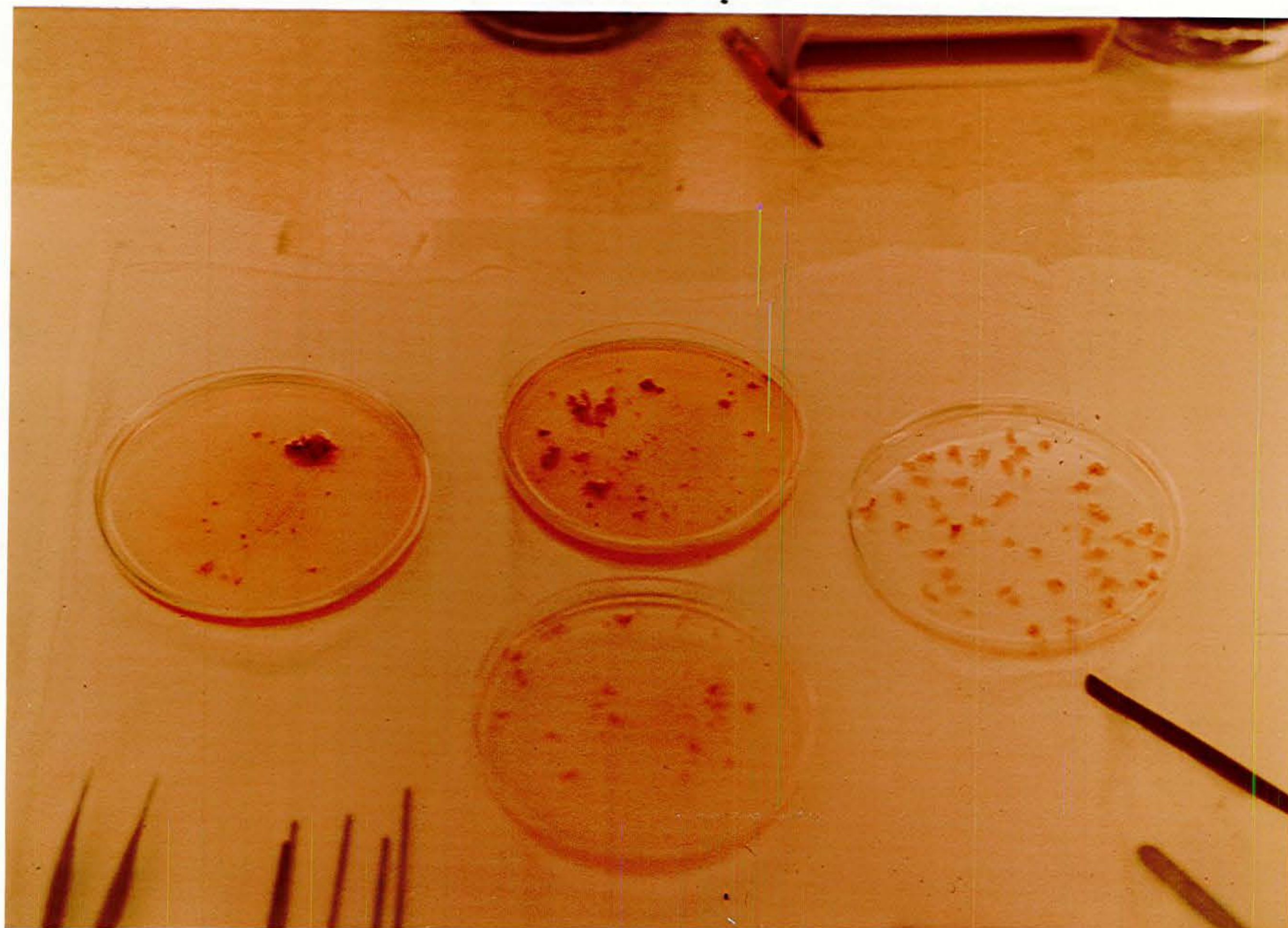

Figure 8. Diced specimens of mammary tumors as prepared for implantation.

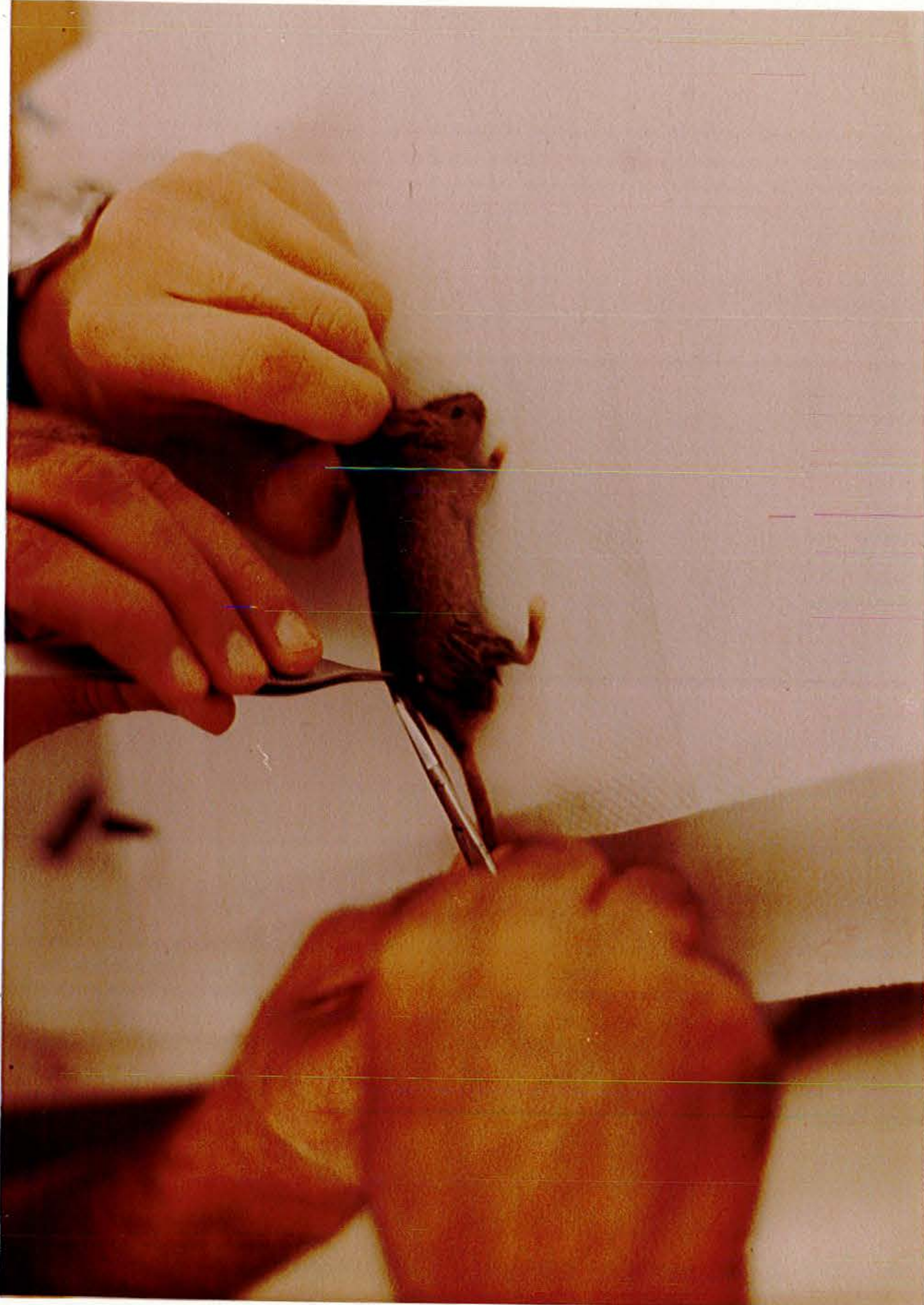

Figure 9. Incision for tumor implantation.

and directed toward the implantation site midway between the left shoulder and left hip in the axillary line (Figure 10). Tumor implantation was completed by introducing a plunger into the barrel of the needle. A clockwise followed by a counter-clockwise rotation of the plunger insured complete evacuation of the tumor. The trocar and its plunger were carefully removed. No sutures were required to repair the skin incision. Tumor samples were randomly distributed by weight within the test groups.

Drug Treatment

Twenty-four hours prior to tumor transplantation, the 54 experimental animals were divided randomly into two groups: 27 as control and 27 which received intraperitoneally 4 mg/kg heparin sodium (128 U/mg) at that time. This is twice the normal anticoagulation dose. The experimental group continued to receive heparin sodium (4 mg/kg) every 12 hours.

Whole Blood Clotting Time Measurement

Using a tapered glass pipette (approximately 10 cm in length), approximately 2.0 ml of blood were drawn from the periorbital venous plexus of the eye. This sample was then placed in a well on a thin glass plate and housed in a water bath at 37°C. The sample was quickly held in a perpendicular position every 30 seconds until a firm clot had formed (87).

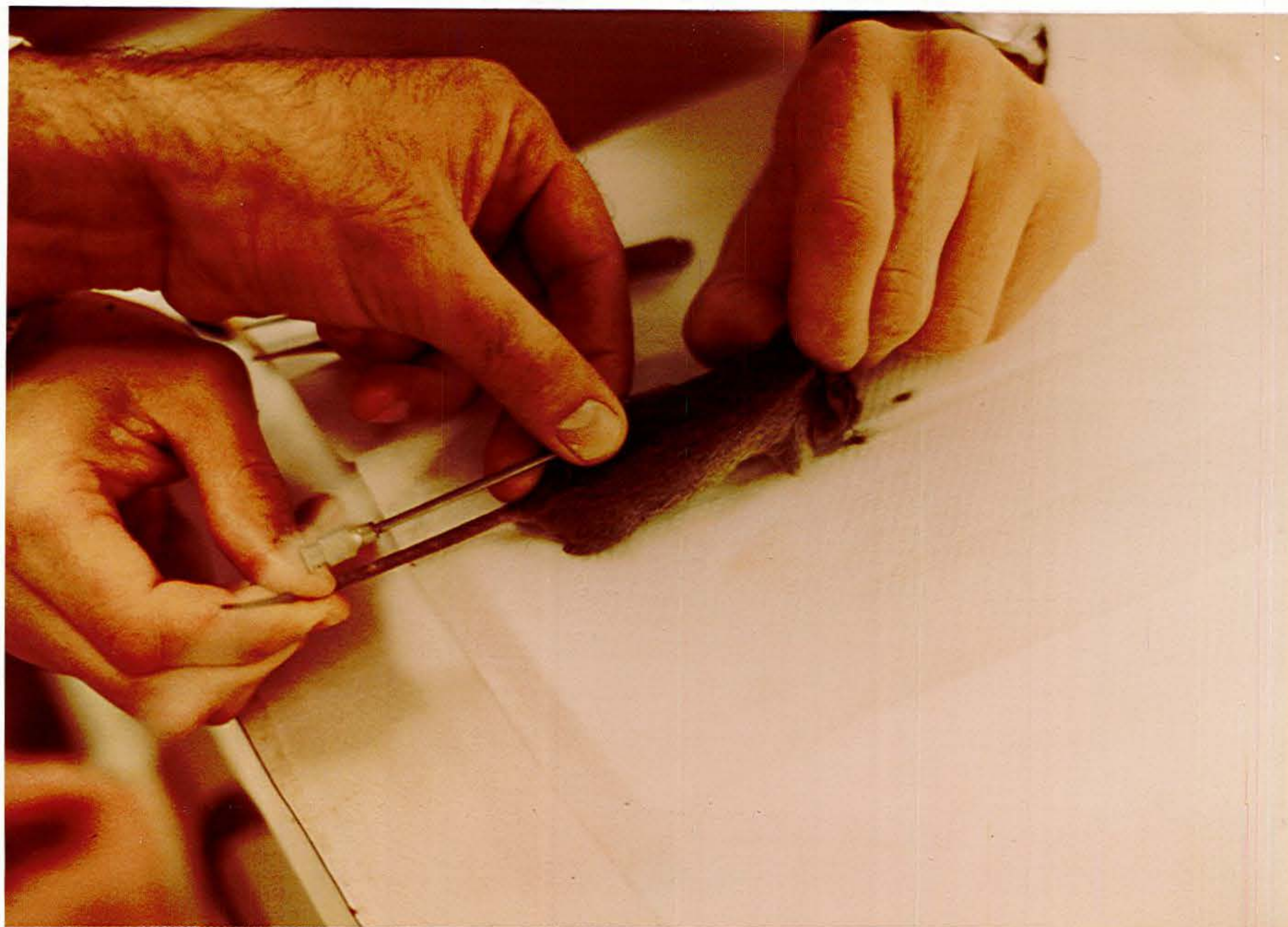

Figure 10. Use of 13-gauge trocar for subcutaneous implantation of the tumor.

Tumor Dissection and Photography

The experimental and control animals were sacrificed, using diethyl ether according to the Experimentation Schedule shown in Table I.

After washing the dorsal skin with 70% ethanol, a midline dorsal incision was made from the sacral area up to the base of the neck (Figure 11). The skin was retracted slowly while it and the tumor were carefully dissected from the underlying back musculature (Figure 12). Both sides of the dorsum were exposed equally for comparison regarding local edema, vascular congestion and tumor appearance (Figure 13). Photographs were taken prior to the removal of the tumor. The tumor was then carefully dissected from the supporting skin tissue (Figure 14) noting its adherence or non-adherence, carefully weighed to the nearest 10^{-4} g using a Sartorius balance (type 2463, Fab. #161122) (Figure 15), and preserved in a 40% formaldehyde solution.

Histology

In the histology group, the tumor was removed with the overlying skin for histological evaluation. All specimens were labeled with time of sacrifice, date, and origin of specimen (control, experimental, histological group). The specimens were then processed by embedding them in paraffin. After sectioning (7 microns thick), slides were stained with hematoxylin/eosin for microscopic study. The slides were prepared in the following manner:

Table I

Experimentation Schedule

Sacrifice time (Implant = 0 hr)	CONTROL (N = 27)				HEPARIN Na (N = 27)			
+6 hrs	X	X	X	X*	X	X	X	X*
+12 hrs	X	X	X	X*	X	X	X	X*
+24 hrs	X	X	X	X*	X	X	X	X*
+2 days	XX	XX	XX	X*	XX	XX	XX	X*
+4 days	X	X	X	X*	X	X	X	X*
+8 days	X	X	X	X*	X	X	X	X*

*Experimental animal selected for histological evaluation.

Figure 11. Dorsal incision prior to tumor dissection.

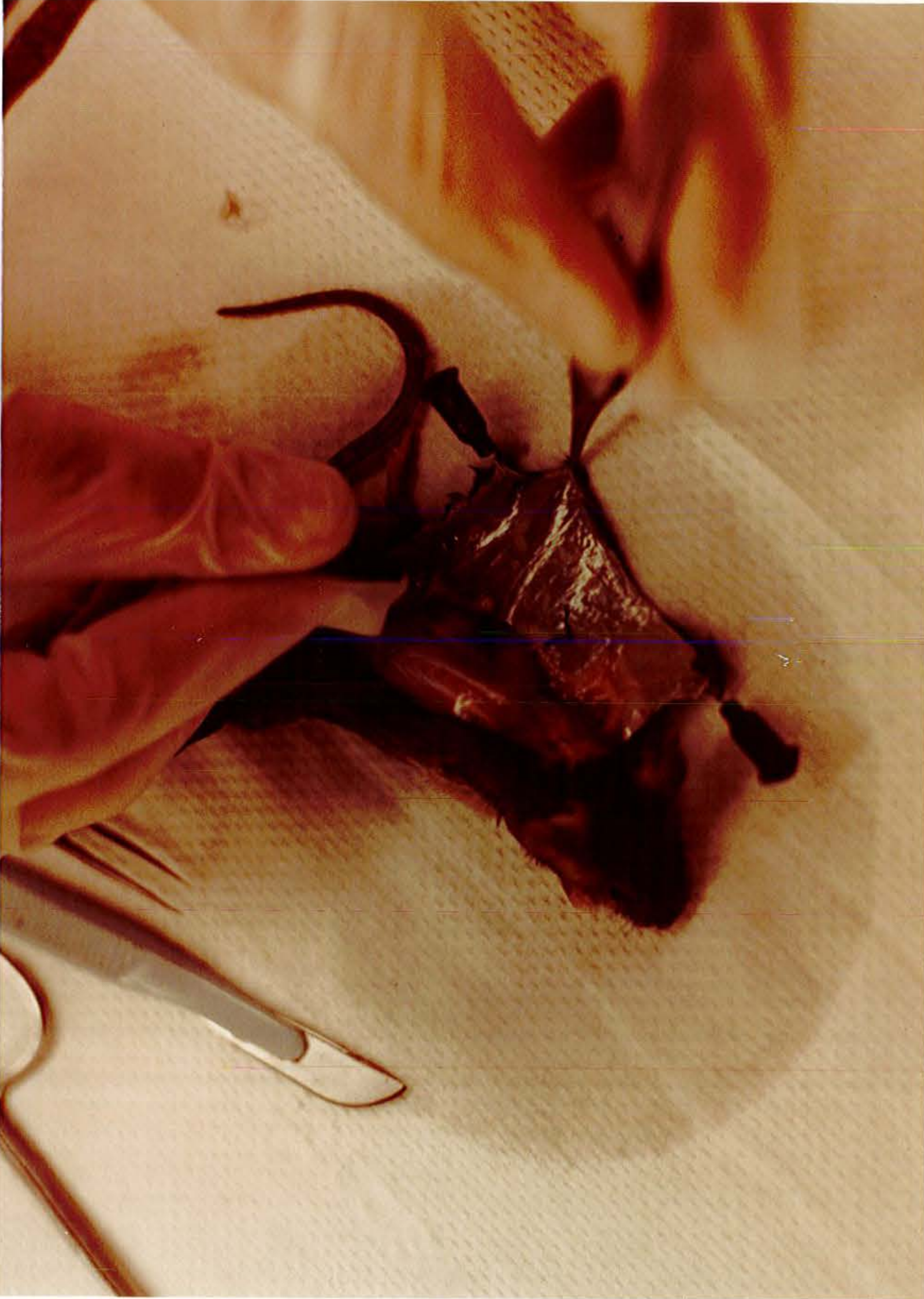

Figure 12. Skin reflection to expose the implanted tumor.

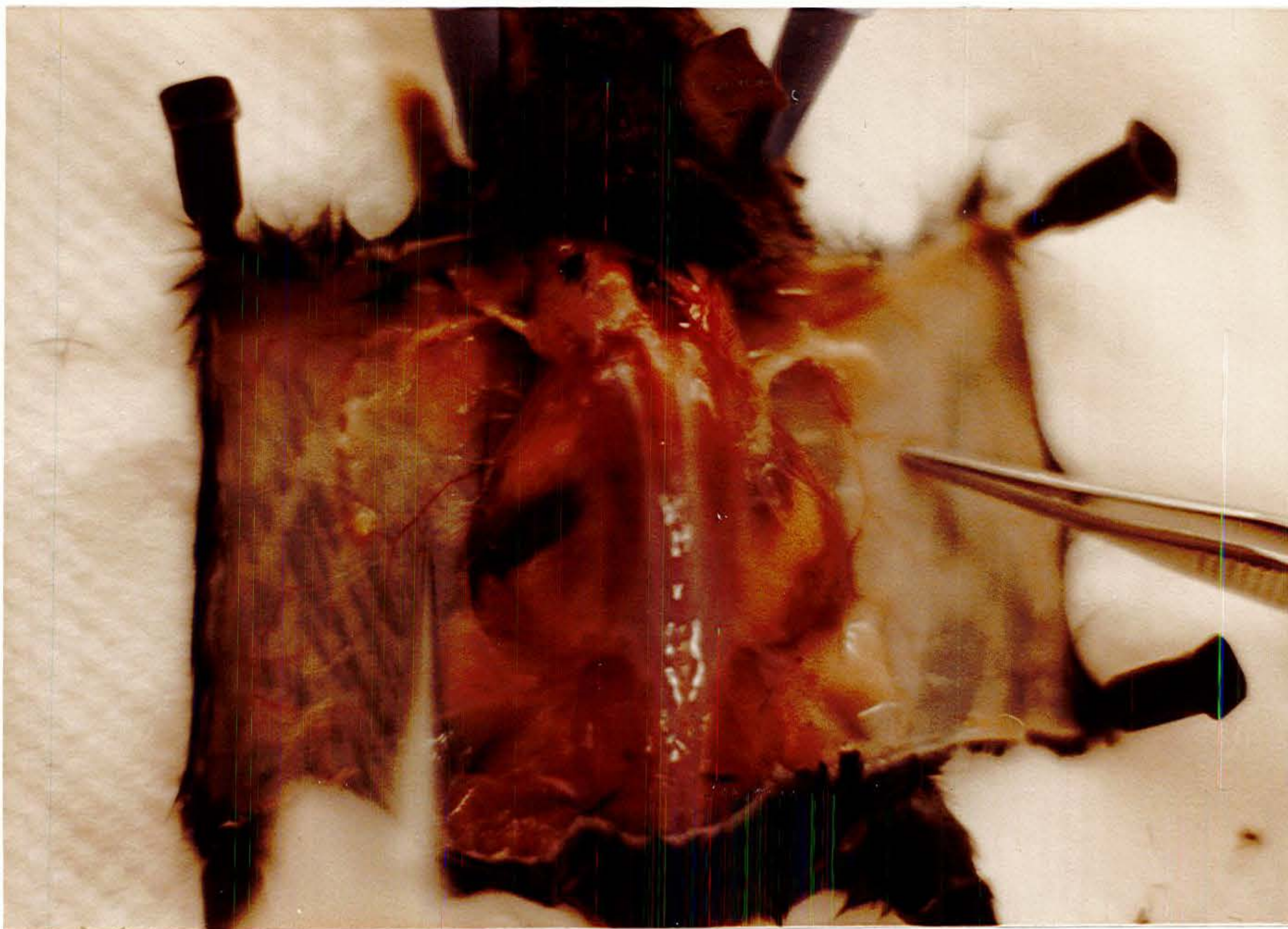

Figure 13. Bilateral skin reflection for comparison. Note the increase in vascular congestion on the tumor side.

Figure 14. Tumor dissection.

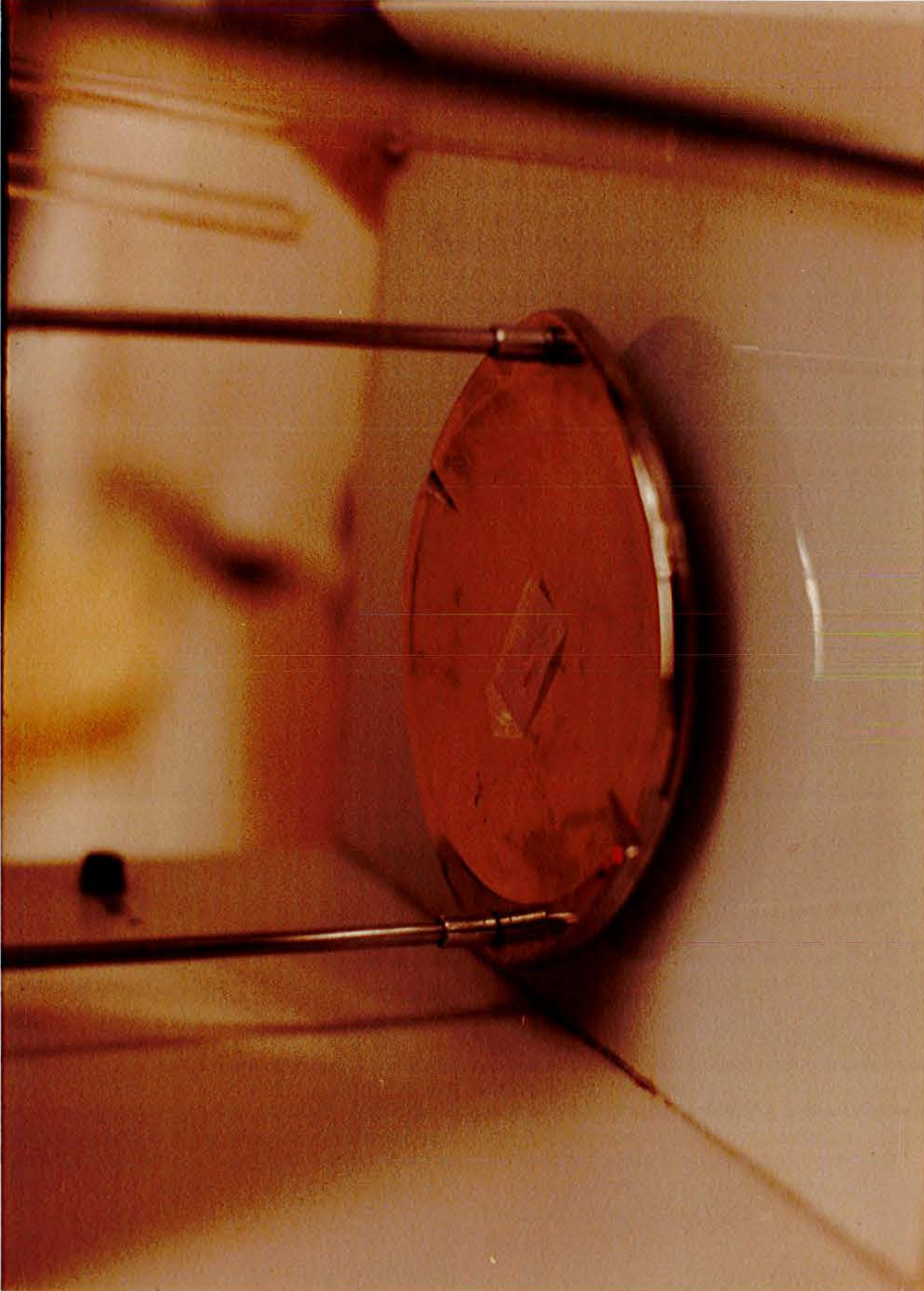

Figure 15. Weighing tumor.

a. After sectioning, a specimen was positioned on a slide and placed on a drying plate at 37°C for 24 hours, then

b. Washed in 70% ethanol, then

c. Placed in xylol 2 min to remove excess paraffin, then

d. Tranferred successively to 100%, 95%, 70%, 50%, and 30% ethanol, 1 min each, then

e. Stained in Harris' hematoxylin (diluted 1:9 with distilled water) for at least 15 min, then

f. Placed successively in 30% and 50% ethanol for 1 min each, then

g. Destained in 70% acid alcohol until sections appear light red on gross inspection, then

h. Washed momentarily in 70% ethanol to remove excess acid and placed in 70% alkaline alcohol until sections became bluish in color, then

i. Counterstained in 0.5% eosin in 95% ethanol for 1 min, then

j. Washed in 95% ethanol only until the excess eosin was removed and the sections appeared purple in color, then

k. Placed in absolute ethanol for not over 1 min (prolonged exposure to ethanol will remove all the eosin), then

l. Cleared in xylol (1 min or longer) and mounted (thin medium no. 1 coverglass), and lastly

m. When sufficiently hardened, cleaned and labeled.

Animal Weight

All animals were weighed to the nearest g prior to the implantation and at the time of sacrifice.

Statistical Evaluation

In many investigations the researcher is primarily interested in discovering and evaluating differences between effects rather than documenting the effects themselves. In the comparison of the group means, the term treatment is used to refer to the basis on which the two groups were differentiated. The goal of this statistical analysis (analysis of variance) was to establish whether or not any difference between the two treatments was significant ($p < 0.05$). A definitive evaluation of the data was then conducted utilizing multi-variate analysis.

RESULTS

Statistical Comparisons

Tables II and III list the raw data obtained while Table IV carries the mean data and corresponding standard error of the mean.

A very highly significant difference in whole blood clotting time was seen between the control and the experimental group (Table V). The average clotting time in the experimental group was prolonged in comparison to the average clotting time in the control group (p = < 0.001). This illustrates the well known anticoagulant action of heparin, the only drug used in this study.

A very highly significant difference (p = < 0.001) in tumor weight was also detected between the two groups (Table VI). This is probably not due to differences in inoculum size, since inoculum size was randomly distributed between the experimental and control groups. There was no significant difference in body weight changes between the control and the experimental groups (Table VII).

In the control group there was a trend toward increasing tumor size with time but this was not statistically significant (Table VIII). In the experimental group tumor size tended to level off after initial statistical growth

Table II

Raw Data for Untreated Group

No.	Time, hrs	Tumor Weight, mg	Clotting Time, min	Body Weight Change, g
1	6	06.5	2.3	-0.2
2	6	10.7	1.1	-0.2
3	6	14.1	1.2	00.3
4	12	14.6	1.5	00.2
5	12	09.2	2.7	00.0
6	12	06.8	2.1	00.0
7	24	06.2	1.3	00.0
8	24	17.8	1.0	00.1
9	24	14.3	1.5	00.0
10	48	04.4	1.9	00.0
11	48	05.5	2.0	00.2
12	48	09.7	1.8	00.0
13	48	09.0	1.6	00.2
14	48	10.3	1.7	00.0
15	48	08.2	2.0	00.0
16	96	09.4	1.5	00.2
17	96	20.7	2.0	00.0
18	96	11.5	1.8	-0.2
19	192	08.1	1.2	00.3
20	192	09.6	2.1	00.2
21	192	09.8	1.2	00.4

Table III

Raw Data for Treated Group

No.	Time, hrs	Tumor Weight, mg	Clotting Time, min	Body Weight, Change, g
22	6	07.5	4.4	00.0
23	6	06.3	1.8	00.0
24	6	05.2	4.4	00.0
25	12	02.8	17.6	00.0
26	12	05.1	2.6	00.0
27	12	01.7	8.0	00.0
28	24	05.2	2.9	-0.2
29	24	05.8	4.3	-0.2
30	24	02.7	3.4	00.3
31	48	02.1	3.3	00.4
32	48	06.3	3.6	00.3
33	48	05.3	2.7	00.4
34	48	06.0	3.2	-2.0
35	48	05.3	3.0	-0.4
36	48	04.8	2.9	00.2
37	96	04.6	3.5	00.3
38	96	04.9	4.2	-0.4
39	96	05.8	5.2	-0.4
40	192	03.7	3.1	00.4
41	192	05.7	5.1	00.5
42	192	06.3	1.1	00.4

Table IV

Mean Values ± 1 Standard Error of the Mean

	Term	Time in Hours					
		6	12	24	48[a]	96	192
Tumor	T	6.330 ± 0.664	3.200 ± 1.002	4.560 ± 0.949	4.960 ± 0.614	5.100 ± 0.255	5.230 ± 0.786
Weight, g	U	10.430 ± 2.170	10.190 ± 2.186	12.760 ± 3.435	7.850 ± 0.971	13.860 ± 2.454	9.160 ± 0.537
Clotting	T	3.5 ± 0.9	9.4 ± 1.7	3.5 ± 0.4	3.1 ± 0.1	4.3 ± 0.5	3.1 ± 1.2
Time, min	U	1.5 ± 0.4	2.1 ± 0.3	1.3 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.5 ± 0.2
Weight	T	0.0 ± 0.0	0.0 ± 0.0	-0.3 ± 0.0	-1.8 ± 0.4	-1.7 ± 0.2	4.3 ± 0.0
Change, g	U	-0.3 ± 0.2	0.7 ± 0.1	0.3 ± 0.0	0.7 ± 0.0	0.0 ± 0.1	3.0 ± 0.1

T = treated group

U = untreated group

[a]Means represent data for three animals, except for the +48 hour values which represent six animals per.

Table V

Analysis of Variance Test Regarding Clotting Time

Term	df	Sum of Squares	Mean Square	$F_{obs.}$	$F_{crit.}$ 0.05	*p*
Between treatments	1	71.501	71.501	16.073	4.12	<0.001
Between time	5	55.095	11.019	2.477	2.48	0.05
Residual	35	174.003	$s^2 = 4.449$			
Total	41	300.599				

df = Degrees of freedom

p = Observed level of significance

Table VI

Analysis of Variance Test Regarding
Tumor Weight

Term	df	Sum of Squares	Mean Square	$F_{obs.}$	$F_{crit.}$ 0.05	p
Between treatments	1	305.640	305.64	33.30	4.12	<0.001
Between times	5	54.649	10.92	1.19	2.48	>0.10
Residual	35	321.22	$s^2 = 9.178$			
Total	41	681.508				

df = Degrees of freedom

p = Observed level of significance

Table VII

Analysis of Variance Test Regarding Body Weight Change

Term	df	Sum of Squares	Mean Square	$F_{obs.}$	$F_{crit.}^{0.05}$	p
Between treatment	1	0.086	0.086	0.55	4.12	>0.20
Between times	5	0.868	0.174	1.12	2.48	>0.20
Residual	35	5.447	s^2 = 0.156			
Total	41	6.401				

df = Degrees of freedom

p = Observed level of significance

Table VIII

Analysis of Variance Within the Untreated Group in Regard to Tumor Weight

Variable	$F_{obs.}$		$F_{crit.}^{0.05}$	*p*
Time	1.236	<	2.90	>0.3
Clotting	0.605	<	3.28	>0.8
Weight	0.702	<	2.90	>0.6

(increase in tumor mass). This again, however, was not a significant difference (Table IX). Also clotting times and body weight changes within each group remained statistically unchanged.

The results were subjected to multiple-variate analysis (ANOVA). From Table X it is obvious that treatment has affected tumor growth significantly ($\underline{p}$ = < 0.0001). Time has no significant effect on tumor weight. There is no significant interaction between treatment and time with reference to tumor weight. Therefore, it is apparent that tumor weight differences are attributable to treatment and not to time.

Table XI demonstrates clotting times are significantly affected by treatment. The treatment group has much longer clotting times than the control group. Duration of treatment has an effect at $\underline{p} = 0.06$ but has no effect at $\underline{p} = 0.05$. In other words, at $\underline{p} = 0.05$ (the traditional probability level for biological research) there is no significant interaction between treatment and duration of treatment with reference to their effect on clotting time; a large number of test subjects would probably produce significant interaction.

Table XII demonstrates no significant difference in body weight changes. Neither treatment nor time has any significant effect on changes in body weight of the animals. It is probable that if this study has been contained for a longer period, duration of treatment may significantly affect change

Table IX

Analysis of Variance Within the
Treated Group in Regard to Tumor Weight

Variable	$F_{obs.}$		$F_{crit.}$ 0.05	p
Time	1.571	<	2.90	>0.2
Clotting	1.745	<	19.43	>0.4
Weight	0.222	<	2.84	>1.0

Table X

ANOVA Multivariate Analysis of the Interaction between Tumor Weight as the Dependent Variable with Treatment and Time as the Independent Variables

Source of Variation	Sum of Squares	df	Mean Square	$F_{obs.}$	$F_{crit.}$ 0.05	p
Main effects	360.289	6	60.238	6.827	2.42	<0.000
Treatment	305.640	1	305.640	34.751	4.17	<0.000
Time	54.649	5	10.930	1.243	2.53	0.314
Two-way interaction	57.364	5	11.473	1.304	2.53	0.288
Explained	417.653	11	37.968	4.317	2.10	0.001
Residual	263.855	30	8.795			
Total	681.508	41	16.622			

Table XI

ANOVA Multivariate Analysis of the Interaction between Clotting Time as the Dependent Variable with Treatment and Time as the Independent Variables

Source of Variation	Sum of Squares	df	Mean Square	$F_{obs.}$	$F_{crit.}$ 0.05	p
Main effects	126.596	6	21.099	4.743	2.42	0.002
Treatment	71.501	1	71.501	16.073	4.17	0.000
Time	55.095	5	11.091	2.477	2.53	0.054
Two way interaction	40.548	5	8.110	1.823	2.53	0.138
Explained	167.144	11	15.195	3.416	2.10	0.004
Residual	133.455	30	4.449			
Total	300.599	41	7.332			

Table XII

ANOVA Multivariate Analysis of the Interaction between Body Weight Change as the Dependent Variable, Treatment, and Time as the Independent Variables

Source of Variation	Sum of Squares	df	Mean Square	$F_{obs.}$	$F_{crit.}$ 0.05	p
Main effects	0.955	6	0.159	0.907	2.42	0.503
Treatment	0.086	1	0.086	0.490	4.17	0.480
Time	0.869	5	0.174	0.991	2.53	0.440
Two-way interaction	0.185	5	0.037	0.211	2.53	0.955
Explained	1.140	11	0.104	0.591	2.10	0.821
Residual	5.262	30	0.175			
Total	6.401	41	0.156			

in body weights.

Macroscopic Comparisons

On gross observation, there was no difference between the control and the experimental group with reference to color, texture and general appearance of the implanted tumor at sacrifice. However, surrounding vascular congestion as evidenced by local vasodilation and local edema appeared to be more pronounced in the control group (see Figures 16 - 19). Also an overlying skin attachment appeared to be more frequent in the control group.

Table XIII summarizes the observed incidence of vasodilation and local edema--parameters which did not lend themselves to precise measurement. As the tumors were being removed, both sides of the skin were exposed. Each animal during dissection was graded a plus or a minus according to the degree of vasodilation. During tumor dissection, local edema was also graded in a subjective manner. In addition, no areas of hemorrhage were seen.

Microscopic Comparisons

Tumor sections from the two groups were evaluated microscopically for the number of viable cells. There appeared to be a greater number of viable cells in the control group (Figures 20, 22, 24, 26, 28, 30). In the experimental group, viable cells were limited to the zonal area at the surface of the tumor (see Figures 21, 23, 25, 27, 29, 31).

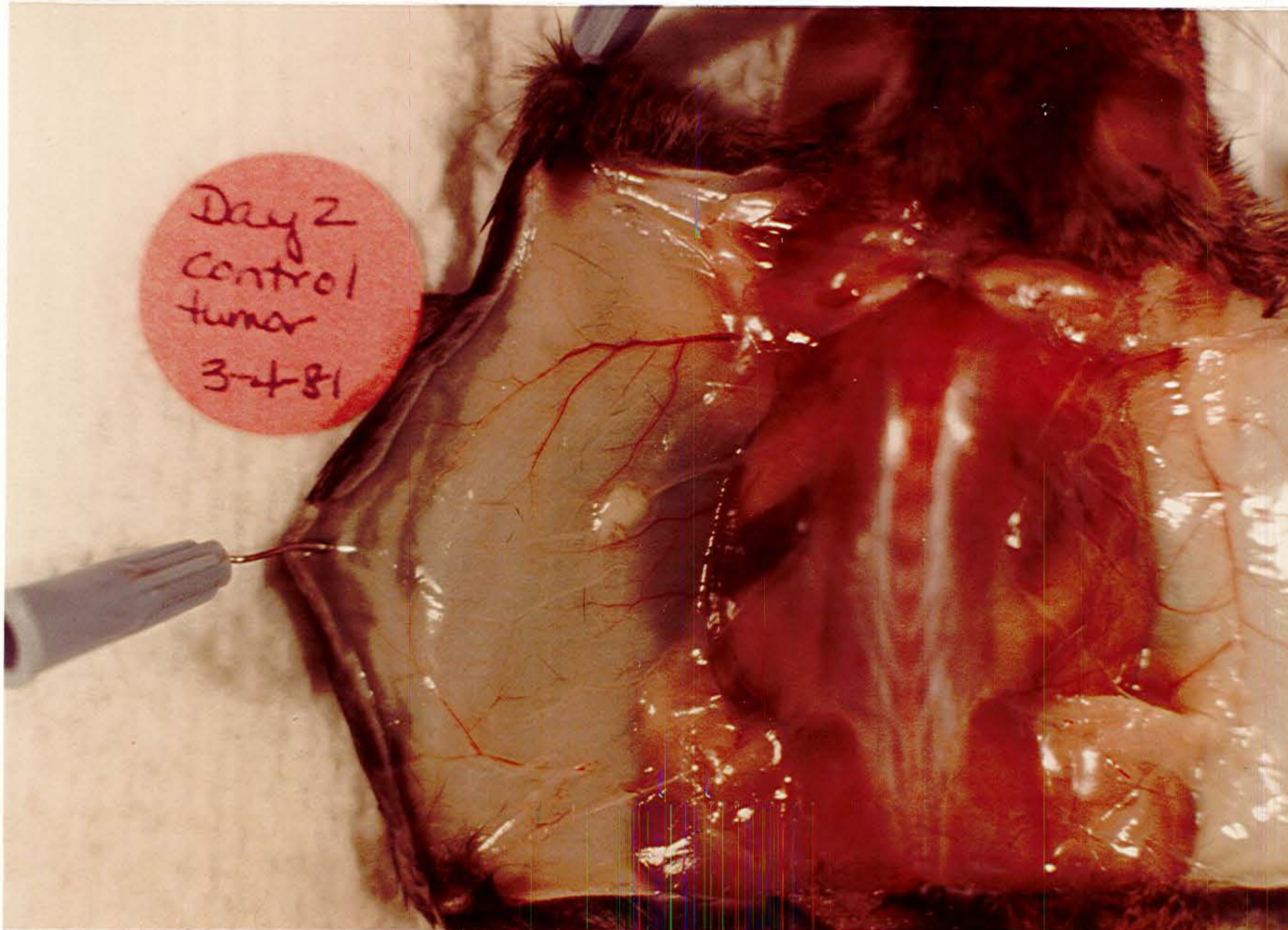

Figure 16. Demonstrating vasodilation in control animals (Day +2). Note the presence of local edema and vessel congestion in the control animal.

Figure 17. Demonstrating vasodilation in experimental animal (Day +2). In the experimental animal cutaneous vessels near the tumor do not show engorgement.

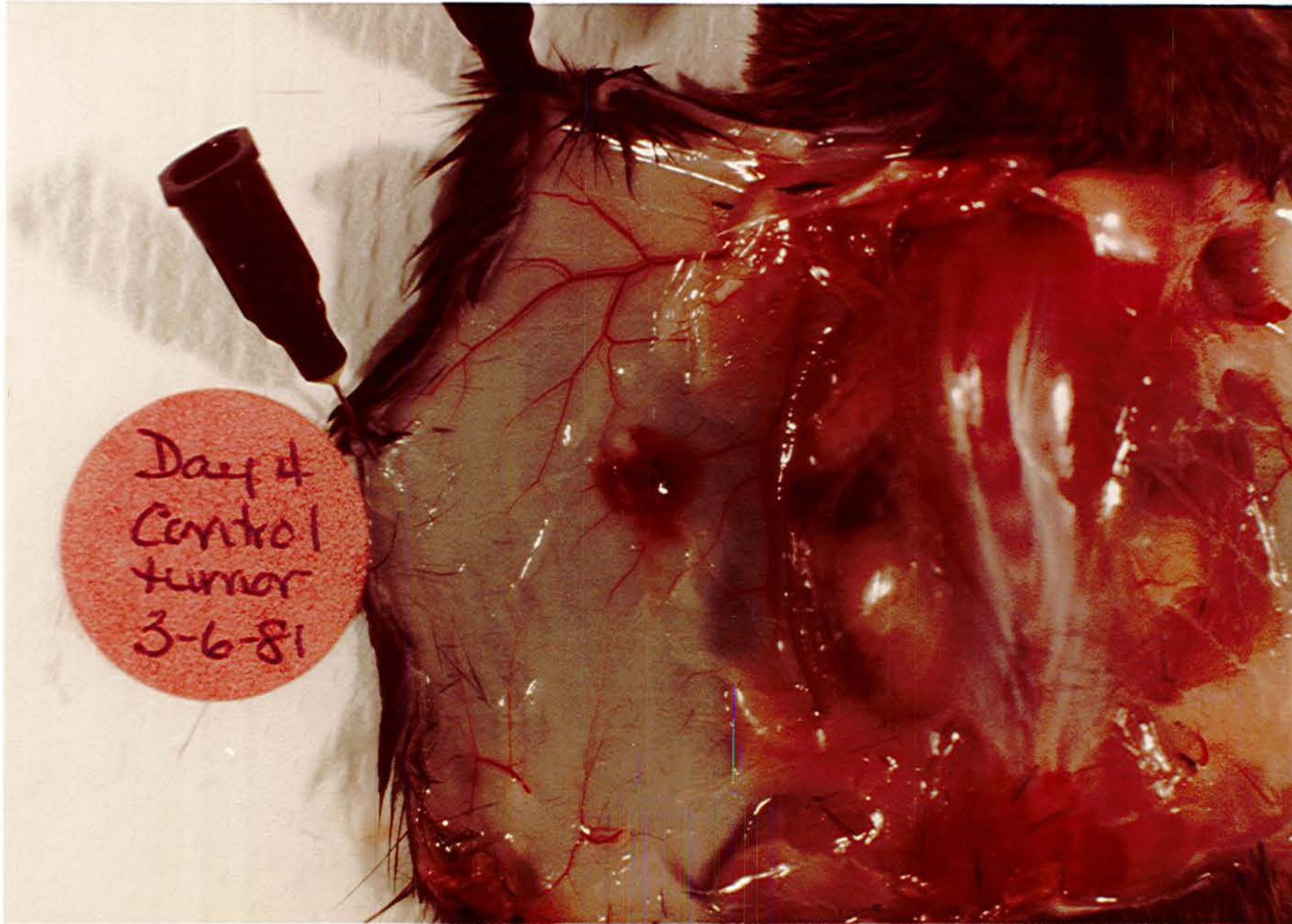

Figure 18. Demonstrating vasodilation in control animals (Day +4). Obvious congestion of overlying skin vessels is observed.

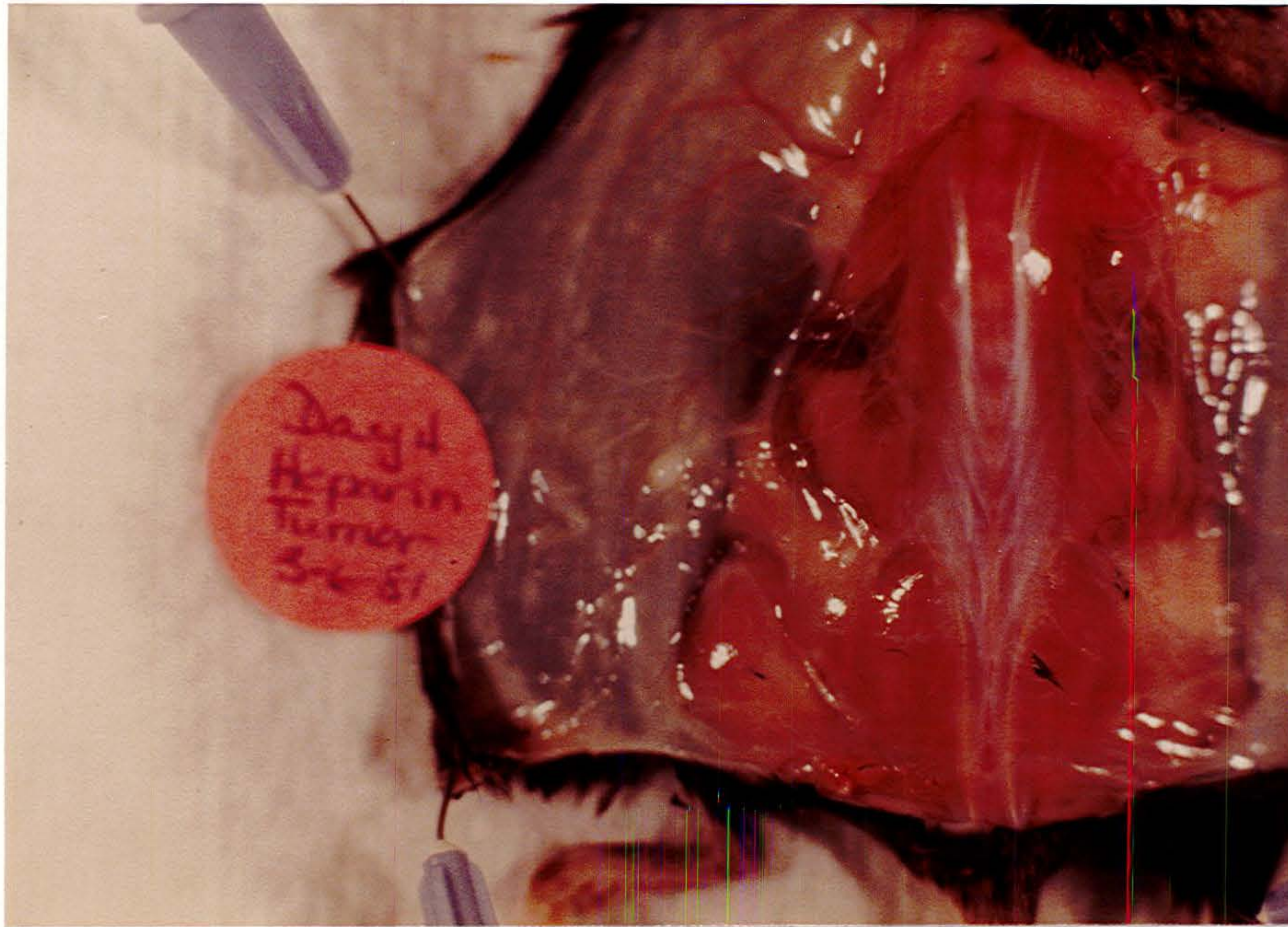

Figure 19. Demonstrating reduced vasodilation in experimental animals (Day +4). Note size of vessels in vicinity of tumor as compared to those in Figure 17.

Table XIII

Quantal Incidence of Rated Factors

		Time in Hours					
		6	12	24	48	96	192
Vasodilation	T	0/3	0/3	1/3	0/6	0/3	3/3
	U	0/3	1/3	2/3	3/6	3/3	3/3
Local Edema	T	0/3	0/3	1/3	2/6	2/3	3/3
	U	0/3	1/3	3/3	3/6	3/3	3/3

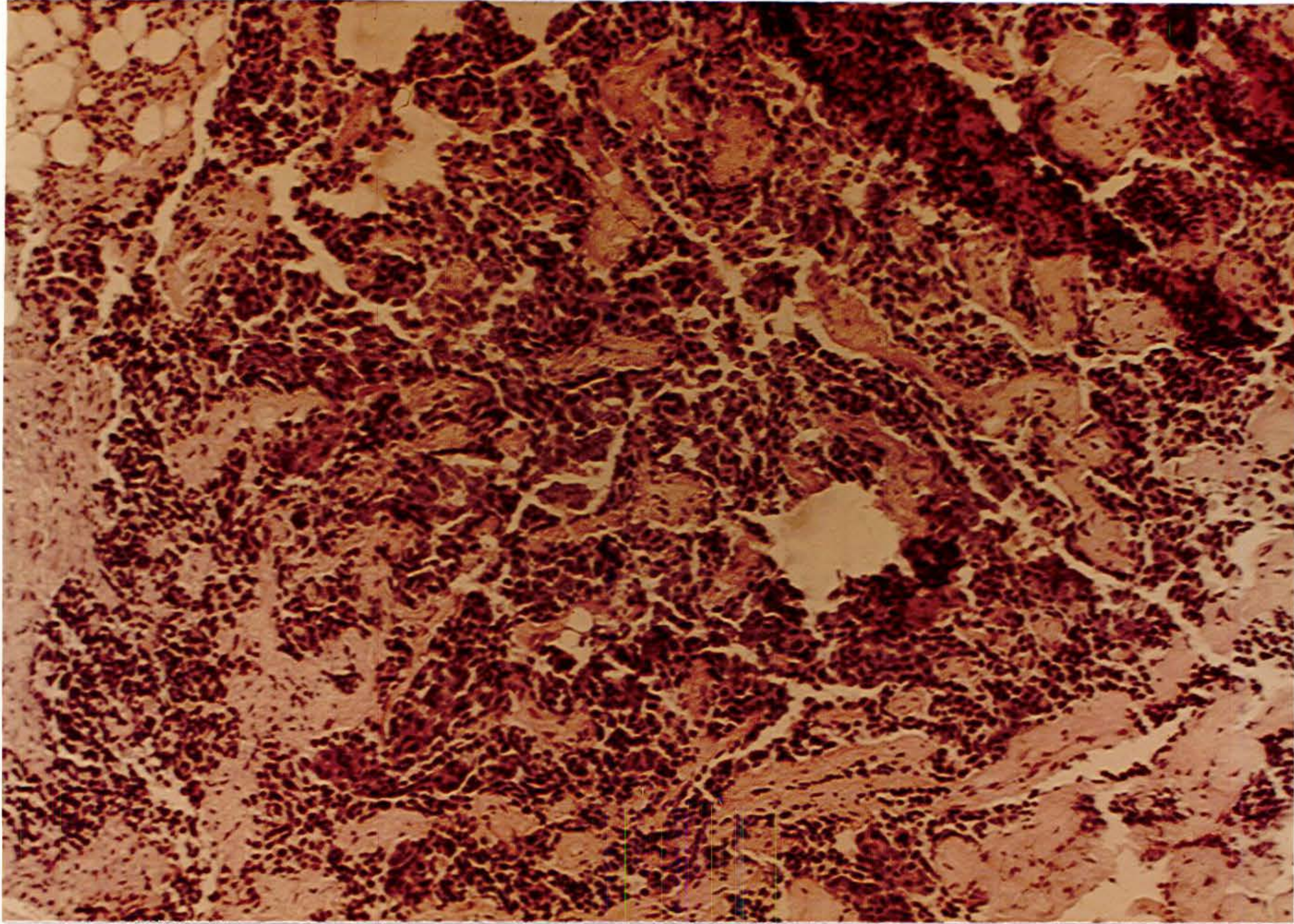

Figure 20. Mouse mammary tumor in control group at 6 hours (25 X). At 6 hours necrotic and viable tumor cells are observed.

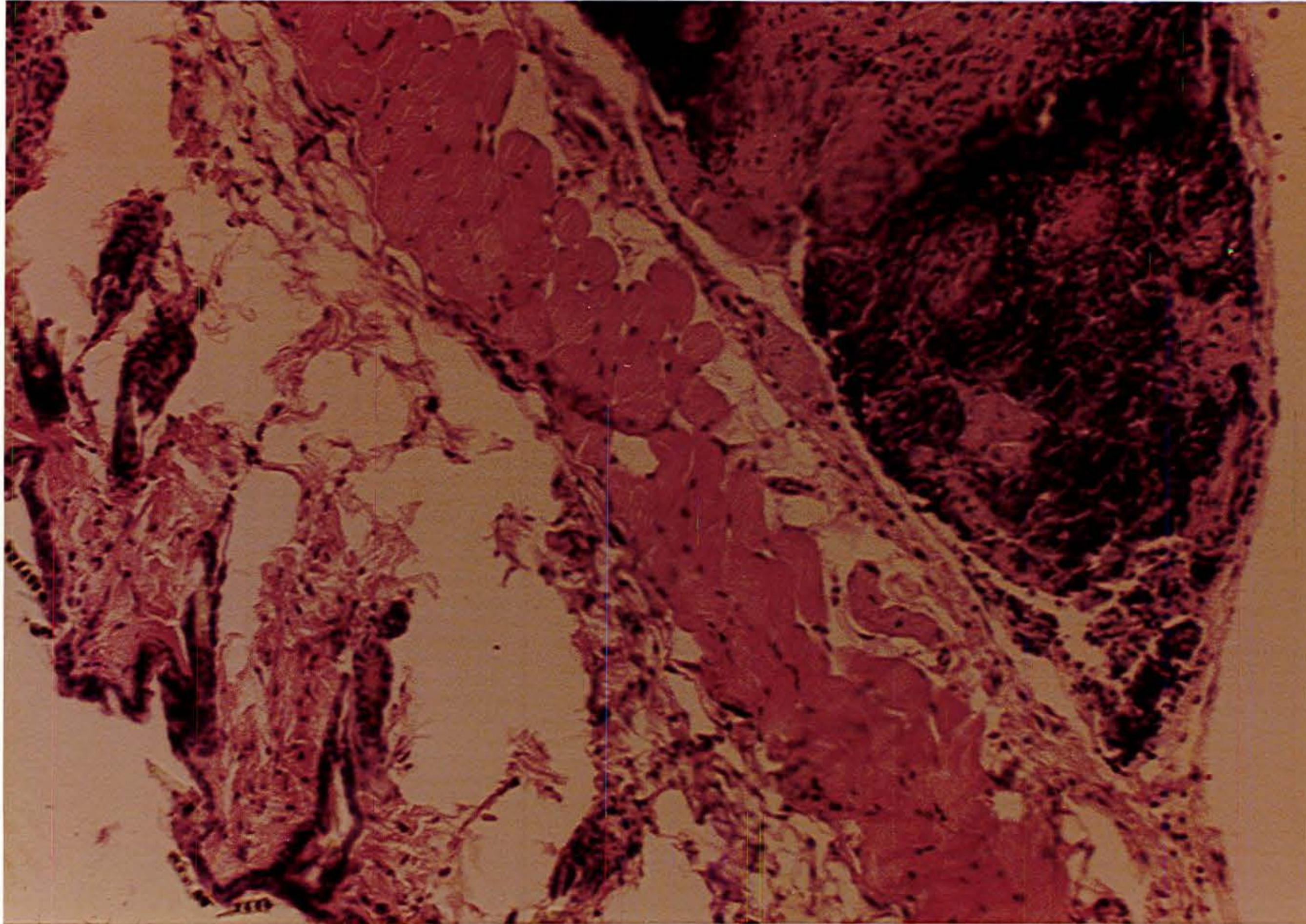

Figure 21. Mouse mammary tumor in experimental group at 6 hours (25 X). Much necrosis is evident throughout the implant.

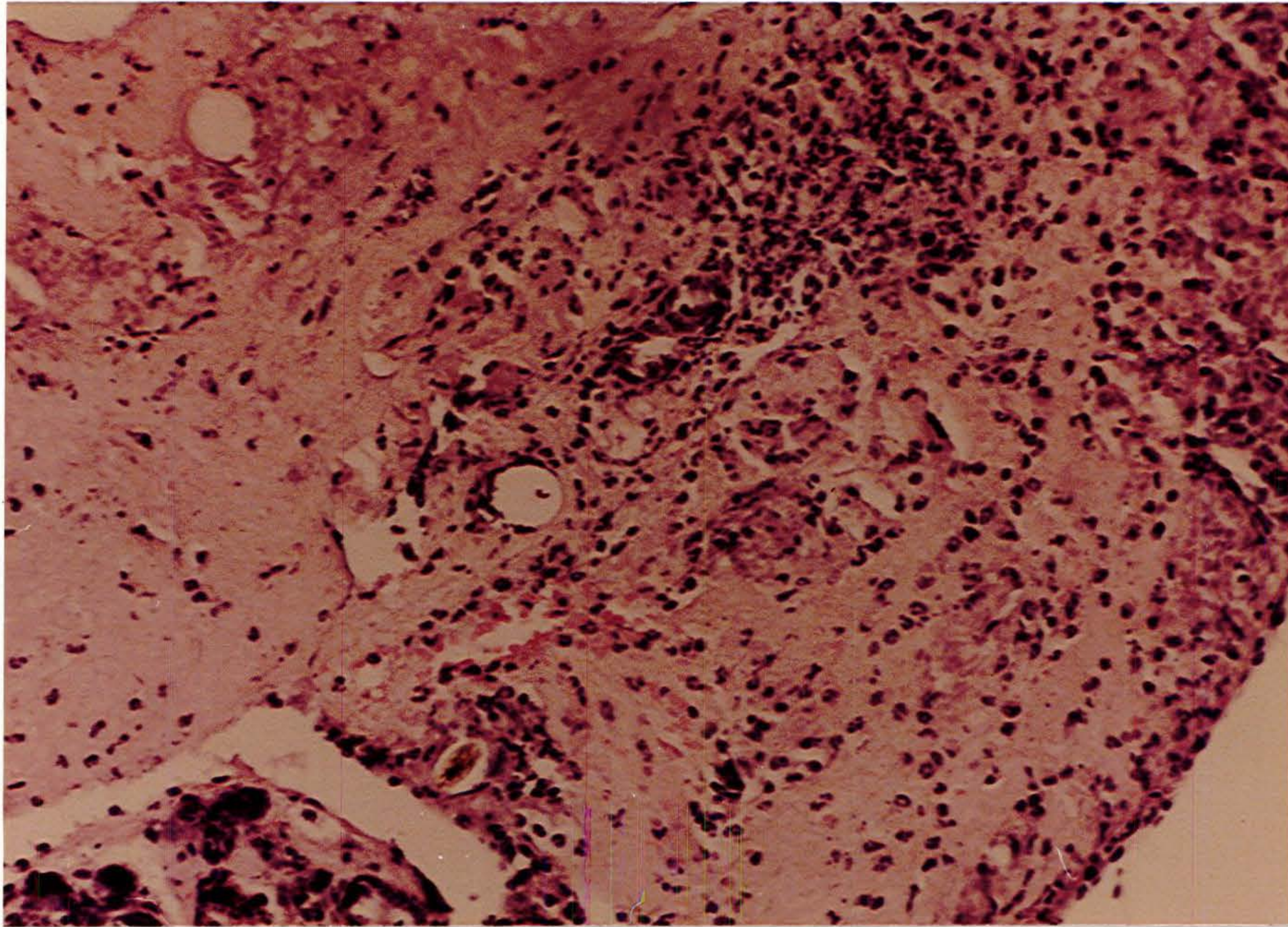

Figure 22. Mouse mammary tumor in control group at 12 hours (25 X). In contrast to Figure 20, an area of central necrosis is observed. Tumor is more cellular and necrosis less evident.

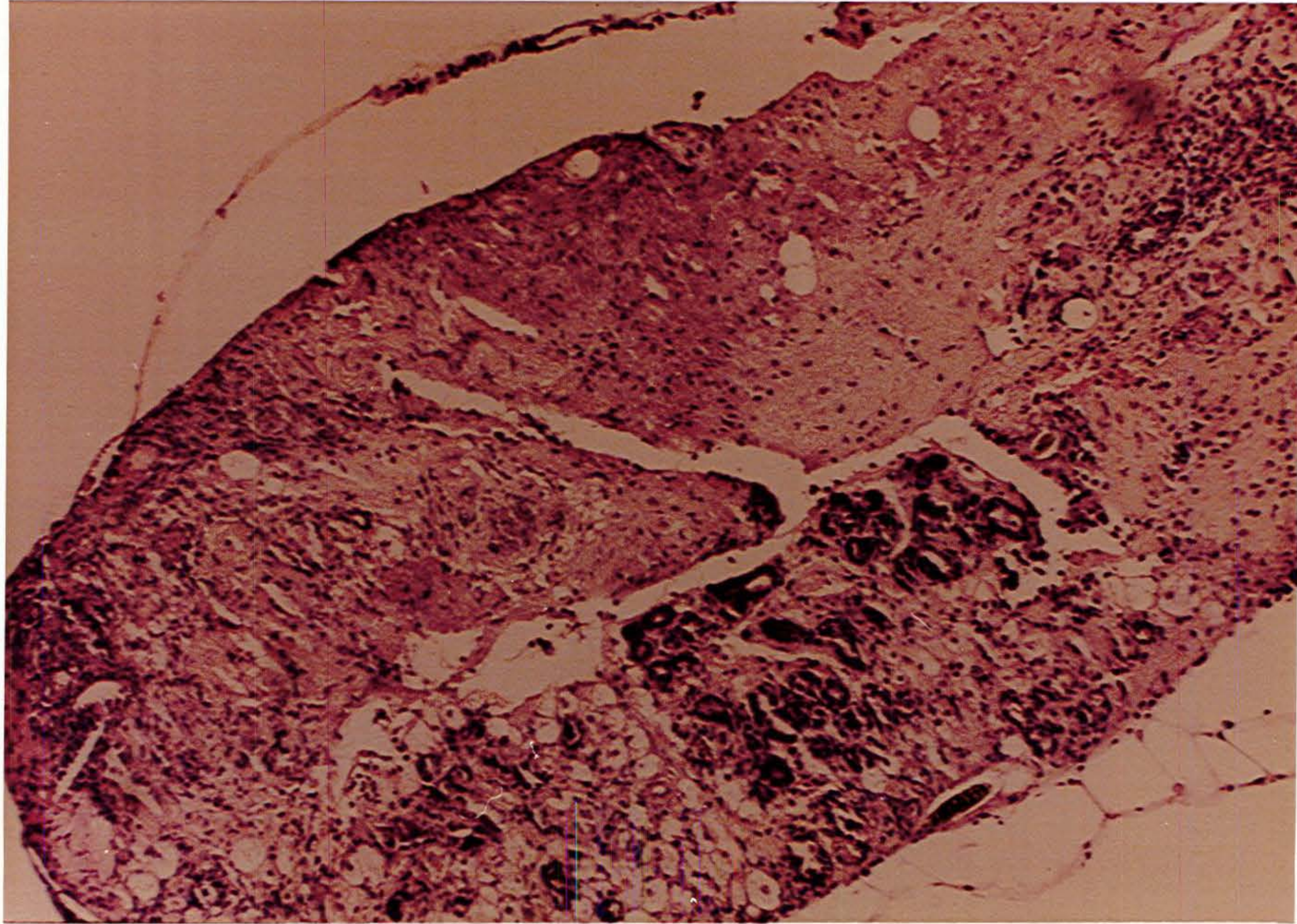

Figure 23. Mouse mammary tumor in experimental group at 12 hours (25 X). Central necrosis with viable tumor cells seen only at the periphery. Note also that PMNs and macrophages appear at the tumor's periphery.

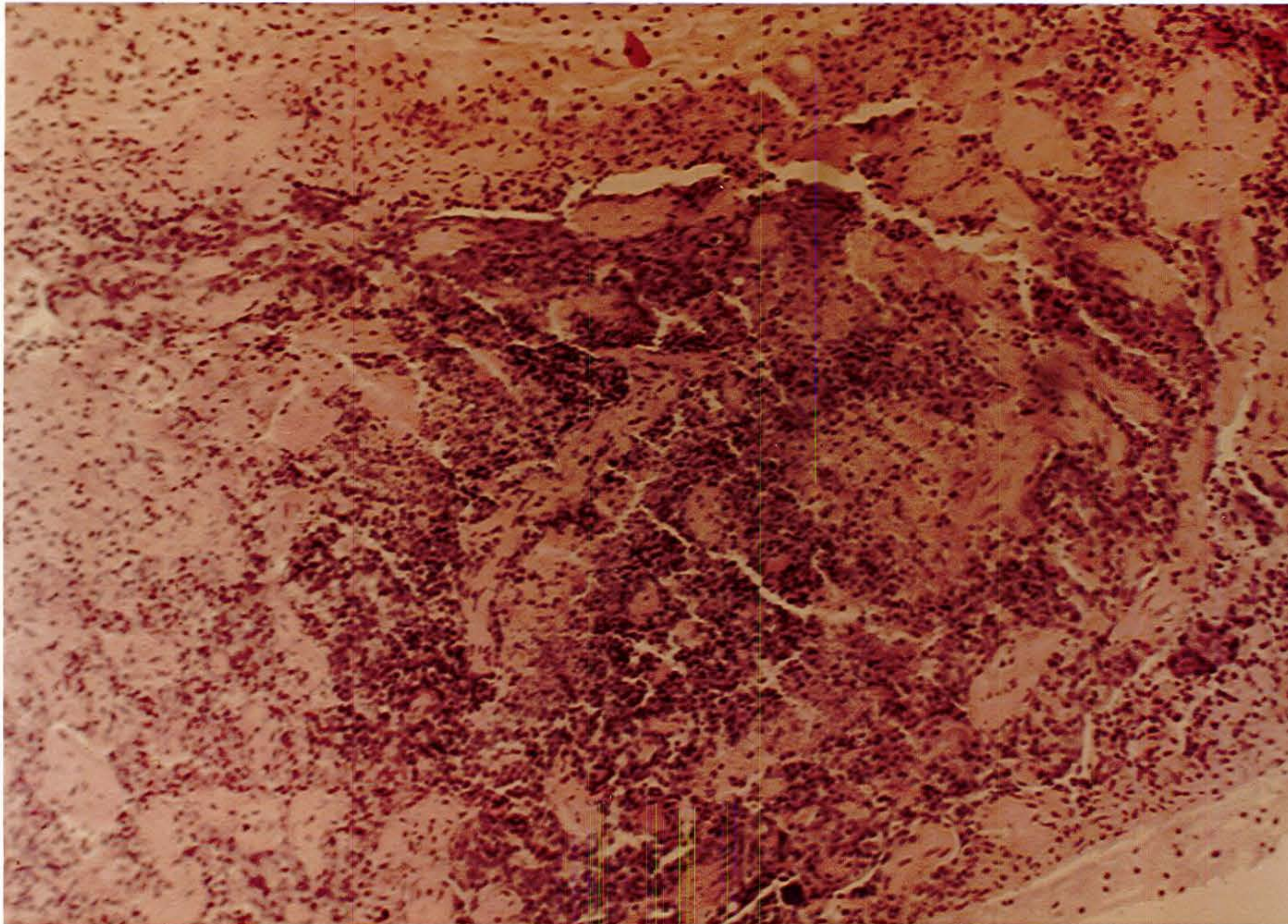

Figure 24. Mouse mammary tumor in control group at 24 hours (25 X). Again note a mass of neoplastic cells with fewer inflammatory elements and a lack of a central necrotic region.

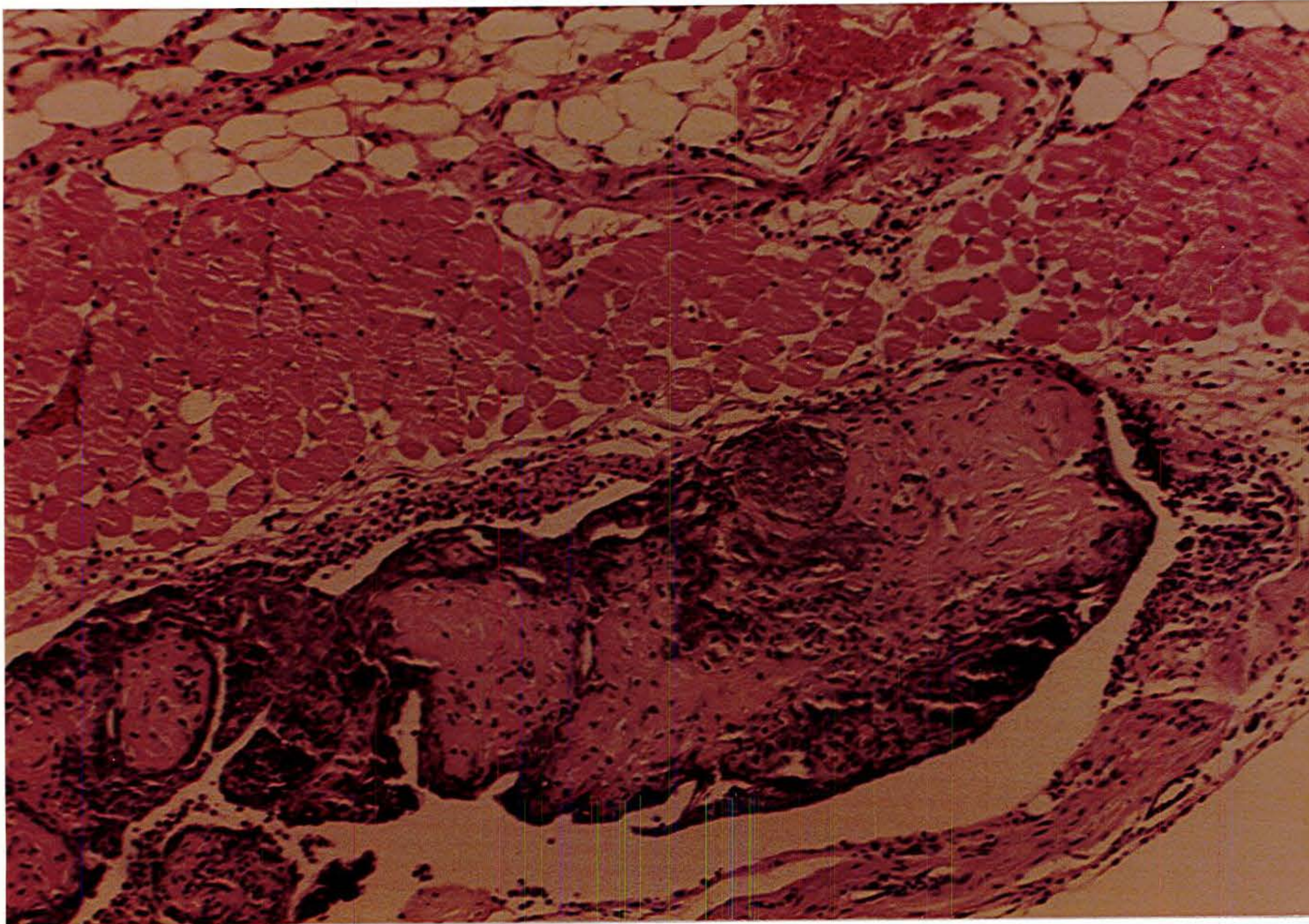

Figure 25. Mouse mammary tumor in experimental group at 24 hours (25 X). A central necrotic area can be observed.

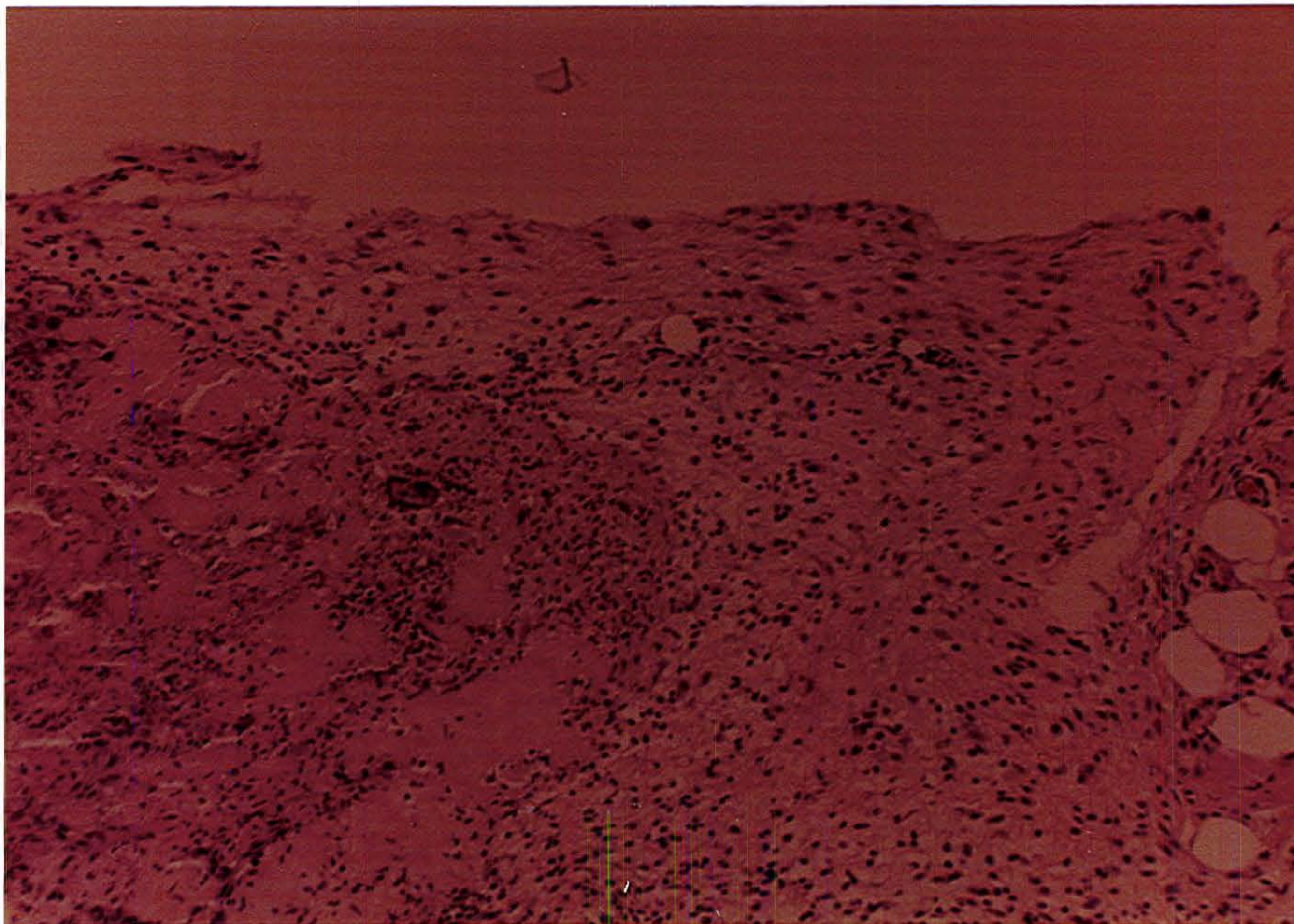

Figure 26. Mouse mammary tumor in control group at 48 hours (25 X). This section is similar in appearance to Figure 20 and Figure 22. Tumor lacks a central necrotic area.

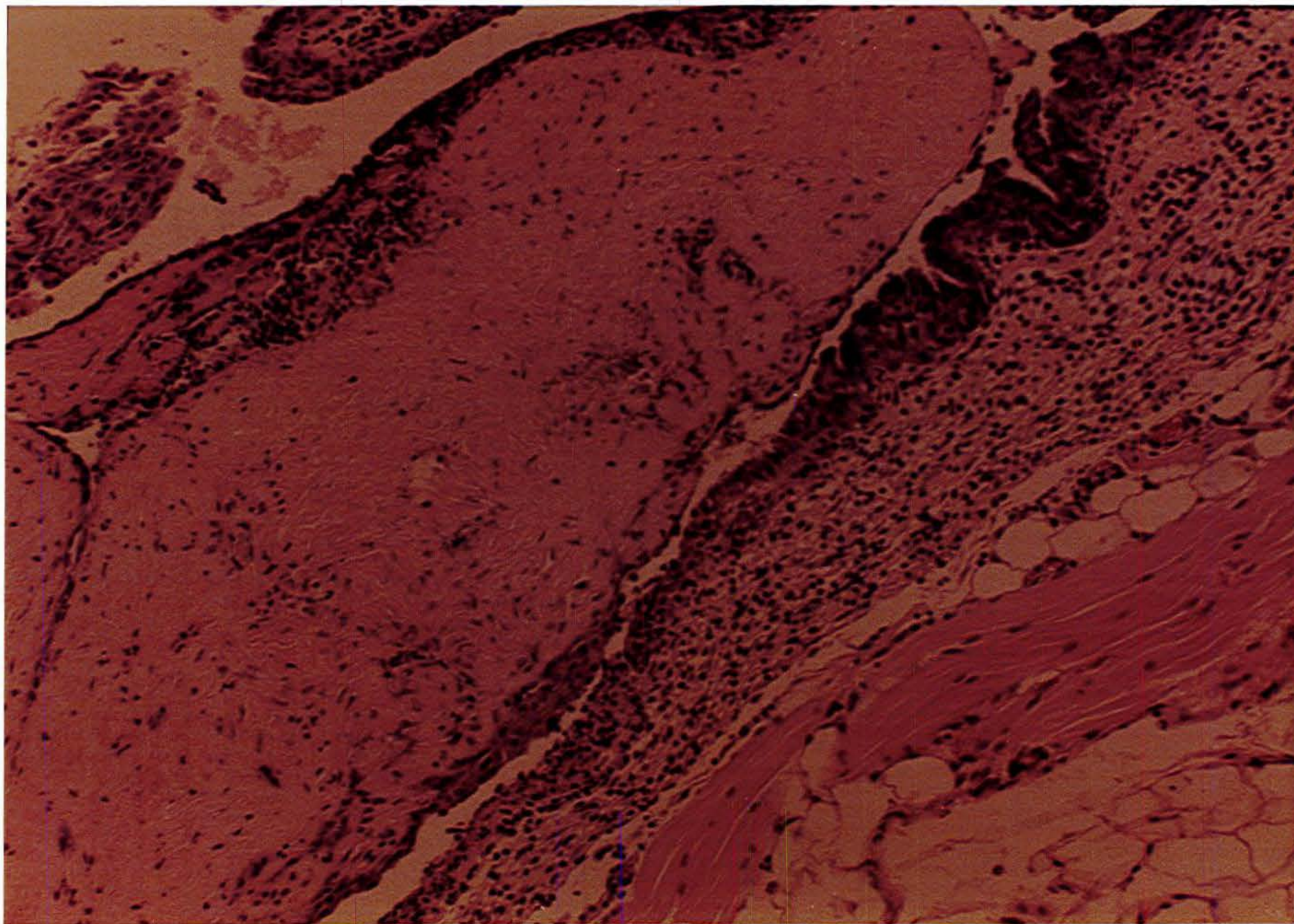

Figure 27. Mouse mammary tumor in experimental group at 48 hours (25 X). A large area of necrosis with very few viable tumor cells can be observed.

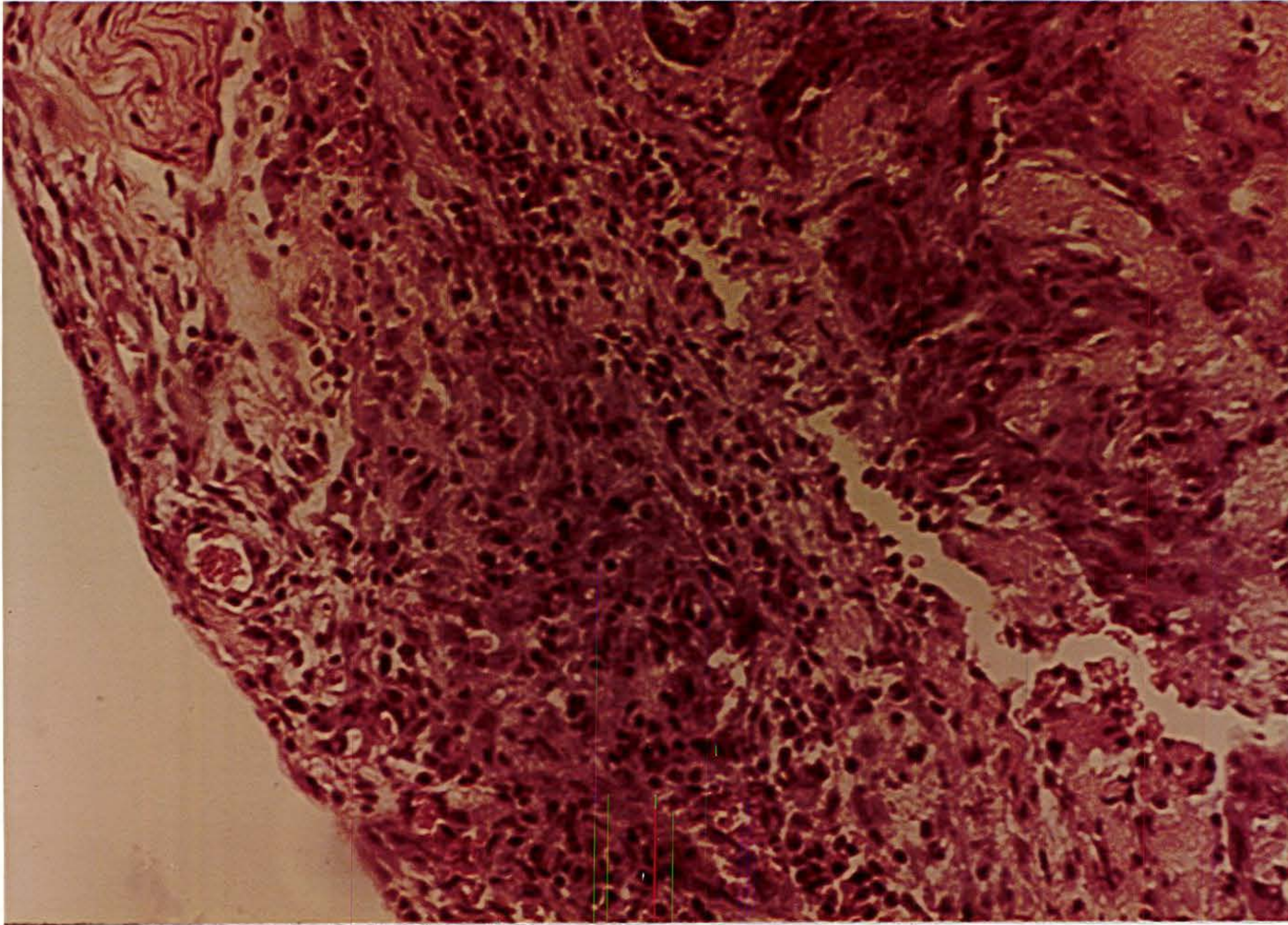

Figure 28. Mouse mammary tumor in control at 96 hours (40 X). At higher magnification, nests of growing tumor cells can be observed.

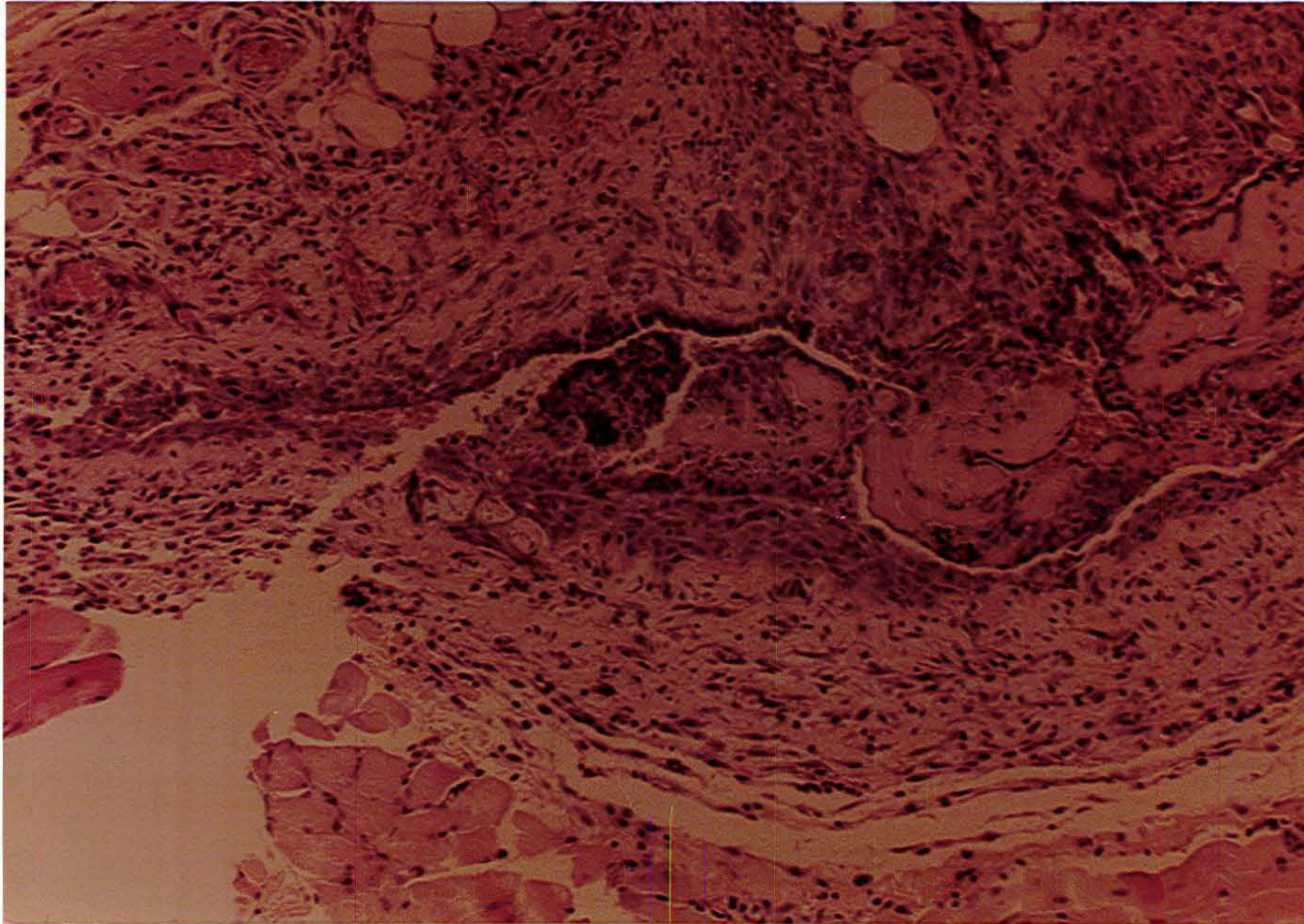

Figure 29. Mouse mammary tumor in experimental group at 96 hours (25 X). Viable tumor cells are observed only at the periphery of the tumor.

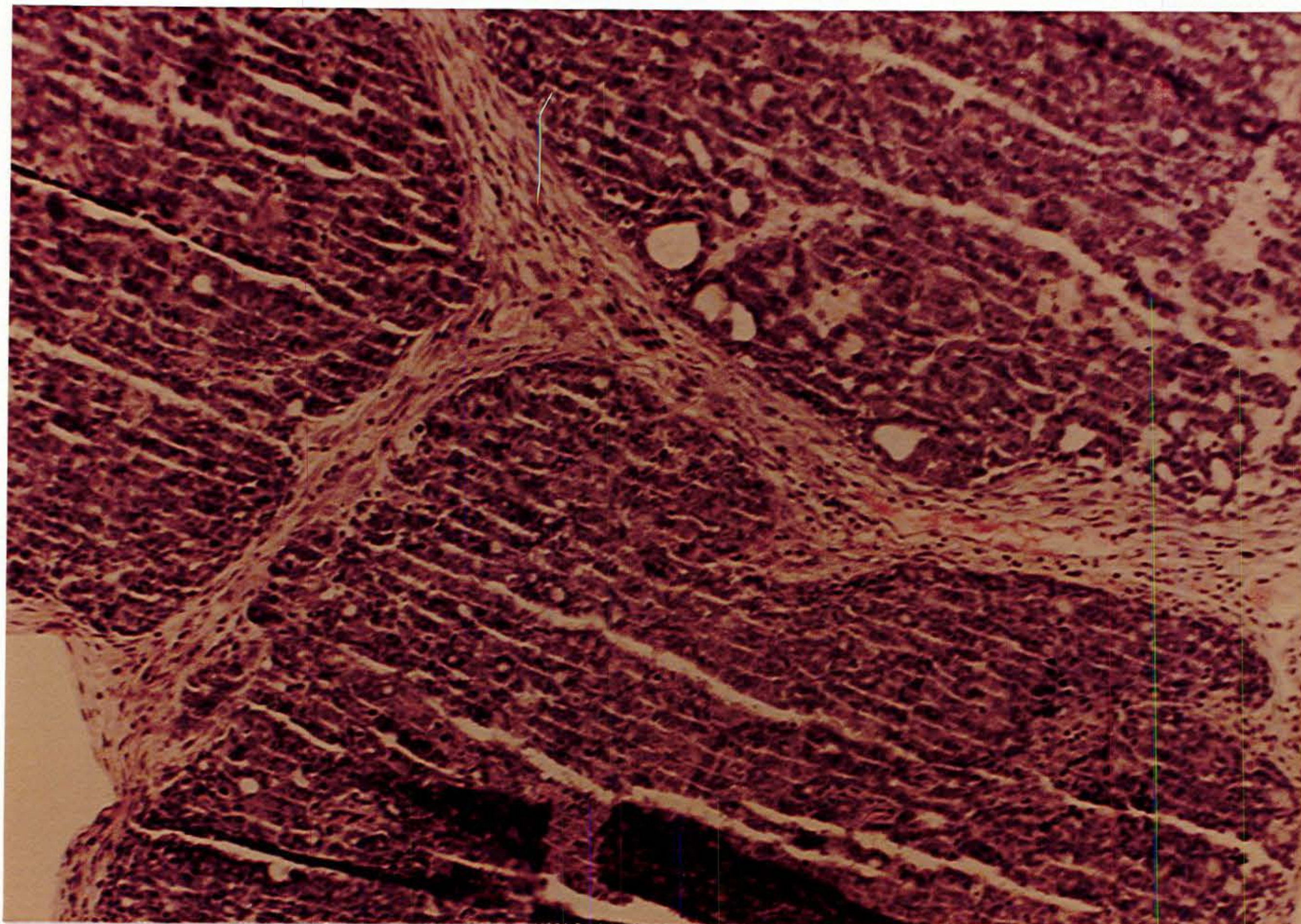

Figure 30. Mouse mammary tumor in control group at 192 hours (40 X). Solid tumor lobules separated by connective tissue with neo-vasculature.

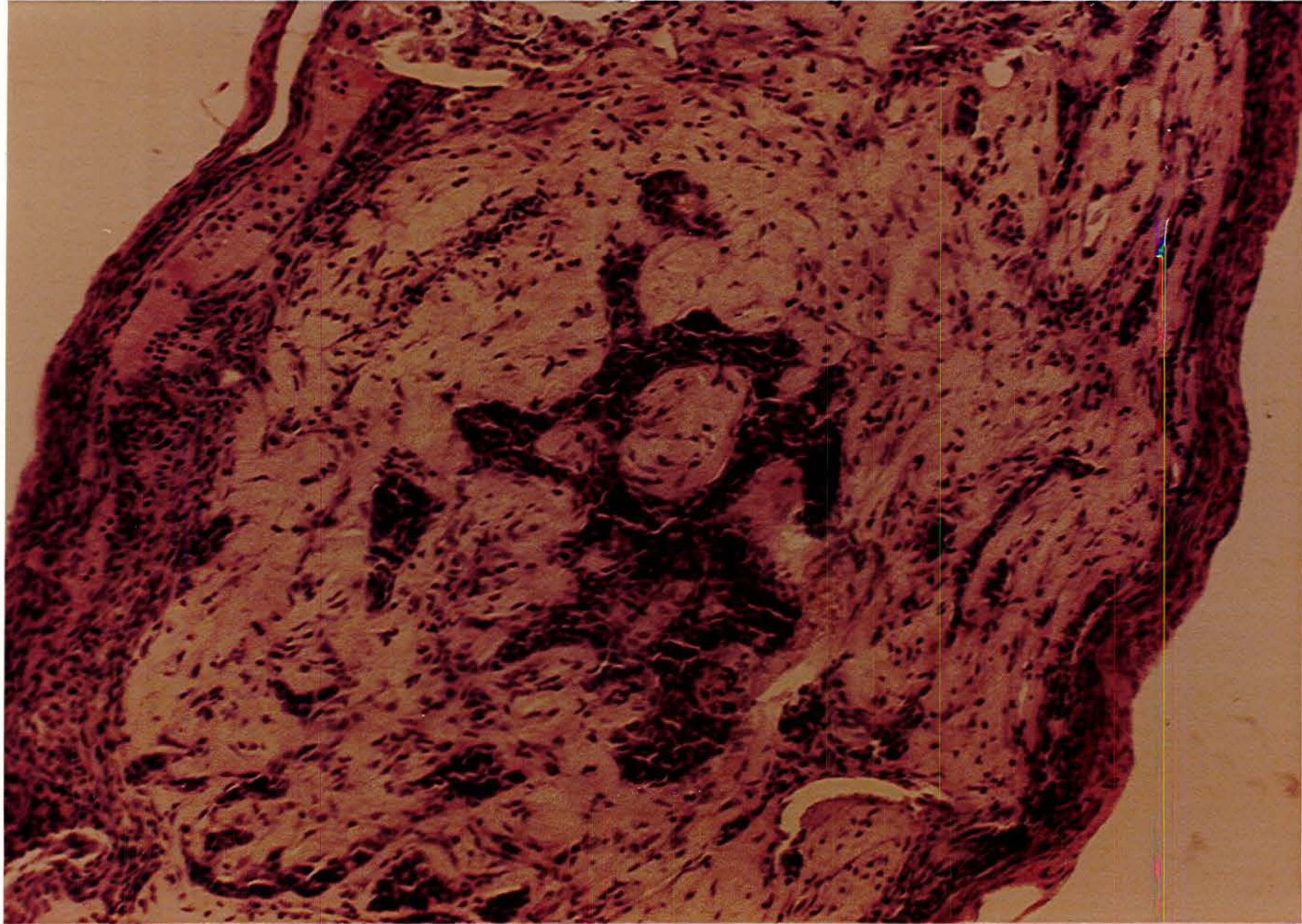

Figure 31. Mouse mammary tumor in experimental group at 192 hours (40 X). A large area of necrosis with relatively low numbers of tumor cells located along the edge of the tumor.

Definite necrosis was seen in most of the histologic sections prepared from experimental animals. However, in a few slides viable cells were seen centrally and these were thought to be the result of the sectioning process (see Figures 23, 31).

Leukocyte infiltration (polymorphonuclears, microphage, lymphocytes) was observed, and a great deal of intragroup variability was seen (see Figures 20-31).

DISCUSSION

This experiment was designed to demonstrate any effects that heparin might have on the growth of implanted mammary tumors in a susceptible, genetically uniform breed of mice.

It is clear from the data collected here that heparin treatment affects tumor growth as early as 6 hours after tumor implantation. This inhibitory effect became progressively more apparent until the time of sacrifice. This favorable response is probably related to heparin's antithrombin activity. The premise could be substantiated by designing a study in which heparin is replaced by an anticoagulant agent with a different mechanism of action such as coumarin. Coumarin primarily affects the synthesis of Vitamin K-dependent clotting factors (serum beta globulins).

The dose of heparin used in this experiment, twice the normal dose, clearly prolonged the clotting time, a necessary consequence of its inhibitory action on thrombin formation. Since the presence of thrombin favors the formation of a stable "fibrin cocoon" for the tumor (49), heparin theoretically should be able to prevent the formation of this presumably protective barrier.

Dvorak has hypothesized that a relationship exists between tumor growth and fibrin cocoon formation (49). If this is true, then chemically interfering with the synthesis of such a structure should result in an altered tumor growth. From this investigation, it is apparent that such a relationship exists although the precise nature of this relationship is not clear. However, the presence or the absence of fibrin was not demonstrated in the present study.

Although weight differences between the tumors of the two groups was the primary focus of this research, less objective criteria such as degree of inflammation, overlying skin adhesion (attachment) and host immune response were also evaluated. For these parameters, differences between the two groups were not large enough to be significant. Of interest was the fact that, in the experimental group viable tumor cells were limited to the zonal area at the surface of the tumor. This can perhaps be explained by the fact that oxygen and nutrients can diffuse into this zone to keep the tumor cells alive.

In 1972, Elias and Bruganolas reported the beneficial effect of anticoagulation in four patients with metastatic carcinoma of the lung not responding to the multiple chemotherapeutic agents alone. After the addition of heparin, excellent clinical responses were seen and confirmed (65). In 1975, Elias, Shukla, and Mink again reported a favorable experience using heparin in patients with metastatic

carcinoma of the lung (63). Fourteen patients not responding to multiple conventional chemotherapy and/or radiotherapy showed significant responses to the same chemotherapeutic agent and/or radiotherapy after heparinization (52, 63).

However, Rohwedder and Sagastume (64) have reported no significant beneficial effect after the addition of heparin to anti-cancer therapy in patients with metastatic carcinoma of the lung.

All of the studies cited above tested heparin in solid tumors. In 1978, Drapkin _et al_. reported on nine patients with acute promyelocytic leukemia who showed favorable responses to heparin therapy (88). More recently, Zacharski _et al_. (60) reported on 25 patients with small cell carcinoma of the lung receiving warfarin in addition to conventional cancer therapy. Median survival in the treatment group was twice as long as in the control or non-anticoagulated group.

The majority of the clinical studies cited above support the hypothesis of this research project that anticoagulants can inhibit tumor growth by inhibiting thrombin formation.

SUMMARY

Fifty-four of the inbred strain C_3H/HeJ Jackson Laboratory female mice approximately one year old and weighing 20-26 grams were implanted with spontaneously occurring mouse mammary tumors and equally divided into control and experimental groups. The experimental group received 4.0 mg/kg sodium heparin (128 U/mg) intraperitoneally on a twelve-hour schedule. Effective anticoagulation was demonstrated by a prolongation of clotting time. Animals (C_3H/HeJ mice) were sacrificed at varying intervals up to 8 days, tumors retrieved and weighed. Definite retardation of tumor growth was demonstrated in the experimental group.

The following conclusions appear justified:

(i) Heparin at the dosage and route used here is an effective anticoagulant agent.

(ii) No major hemorrhagic accidents were seen in the anticoagulated mice at the dose used.

(iii) Implanted mouse mammary tumor growth was inhibited by heparin.

(iv) Polymorphonuclear, macrophage, T lymphocyte and tumor cell necrosis appeared to be too

variable for specific conclusions to be drawn.

(v) Overlying skin attachment (adhesion), blood vessel congestion (dilation) and the presence of local edema appeared more pronounced and occurred more frequently in the control group.

The author agrees with Zacharski (60) that anticoagulant therapy may have an important role to play in the treatment of solid tumors such as mouse mammary adenocarcinoma. Further investigation will be necessary to confirm these findings and to confirm that the beneficial effect of heparin is exclusively due to its antithrombin activity. Experiments utilizing a pharmacologically different anticoagulant (e.g. warfarin) would be especially meaningful.

The present study limited heparin treatment to a maximum term of 8 days. It would be useful to study the effect of heparin for much longer periods. End points for such long term experiments could be animal death or the complete disappearance of the implanted tumor mass.

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