ADVANCES IN CANCER RESEARCH

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ADVANCES IN CANCER RESEARCH

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RENAL CARCINOGENESIS

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I. Introduction

Human renal tumors are comparatively uncommon. Waterhouse (1974) for the Birmingham (England) region gave an incidence of 4.7 and 2.8 per 100,000, which represented 1.5% and 1% of all cancers in males and females, respectively, and Bennington (1973) indicated that malignant renal tumors represented 1.2% of all malignancies exclusive of skin cancer. Those figures compare closely with the observation of Case (1964) that, in England and Wales in 1962, renal tumors accounted for 1.5 and 1.1% of all deaths from neoplastic disease. The etiology of such tumors is obscure, but, except in the younger age group, they are more common in males than in females in practically every country of the world. Geographic differences are present since the incidence is higher in countries such as New Zealand, Denmark, Norway, and Scotland and less in Eire, Italy, Japan, Spain, and Venezuela; others, including Australia, France, Belgium, Netherlands, England and Wales, and the United States are in an intermediate position (Case, 1964). In the United States, there is a cluster of states with relatively increasing death rates from renal tumors and another group with a relatively decreasing death rate from the same cause (Burbank, 1971). Dukes (1964) suggested that incidence is higher in Africa than in other countries. Although it is accepted that tumors of the bladder and renal pelvis are associated with occupational hazards (Dukes, 1964) and with drug abuse (Johansson *et al.*, 1974), there is little evidence of such influences on the etiology of renal tumors. There are a few reports on possible

hereditary influence with those tumors (Brinton, 1960; Riches, 1963; Steinberg *et al.*, 1972), but it is difficult to incriminate genetic rather than environmental factors.

Since little is known about the etiology of human renal tumors, it may be rewarding to examine the mechanism of tumor induction in experimental animals, and the following review considers the various agents that have been shown to cause renal tumors in different species of animals.

II. Spontaneous Renal Tumors in Relevant Species of Animals

Before discussing experimentally induced renal tumors, it is obviously necessary to consider the incidence of spontaneously occurring lesions in the species of animals used for experimental work.

In the rat, McCoy (1909) recorded the presence of 11 renal tumors from almost 100,000 animals autopsied; and in almost 32,000 rats of 8 strains, Curtis *et al.* (1931) described 6 renal neoplasms. In Wistar rats, Ratcliffe (1940) autopsied 468 animals and found 273 with tumors which included 5 renal tumors—4 embryonal-type tumors and a fibrosarcoma. Guérin (1954), from a total of 567 tumors from 16,500 rats, recorded 5 of renal origin; Crain (1958) and Gilbert and Gillman (1958) each noted 3 renal neoplasms from a total of 786 and 1342 Wistar rats, respectively. Although not exhaustive, those examples are sufficient to indicate that renal tumors are uncommon in the rat. In mice, renal tumors are extraordinarily rare (Dunn, 1949). That statement bears out the reports of Haaland (1911) and Slye *et al.* (1921), who found 2 renal tumors from 333 tumors examined and 12 tumors (5 epithelial tumors and 7 sarcomas) from 33,000 autopsies, respectively. In a resumé of 21 reports on neoplasia in mice, Horn and Stewart (1952) listed 12 primary renal tumors, excluding lymphomas, thus confirming the fewness of such lesions. After study of 301 hamsters, Fortner (1957) reported the presence of one Wilms'-like tumor in one of the animals. In addition to those reports on spontaneous tumors in rats, mice, and hamsters, consideration must be given to large numbers of control animals utilized in the various experiments, described later, in which renal tumors were few in number or, mainly, absent.

III. Experimentally Induced Renal Tumors

A. Hormones and Hamster Renal Tumors

1. *Induction*

The first report of an estrogen-induced renal tumor in the hamster was by Vasquez-Lopez (1944), who described in the kidney of an estro-

gen-treated hamster a tumor that he thought was a metastasis from a lesion in the pars intermedia of the pituitary. However, in view of subsequent findings reported by Matthews *et al.* (1947), Kirkman and Bacon (1950, 1952a,b), Horning and Whittick (1954), Horning (1956a,b), Bloom *et al.* (1963a), Manning *et al.* (1964), and Hamilton *et al.* (1974), among others, there is little doubt that Vasquez-Lopez had described a primary renal tumor induced by estrogen treatment.

Initially, it had been reported that estrogen treatment induced renal tumors in intact or gonadectomized males (Matthews *et al.*, 1947; Kirkman and Bacon, 1952a,b), but later it was found possible to induce the lesions in females under circumstances of low progesterone levels (Kirkman, 1957, 1959). Such a state was achieved (1) by ovariectomy; (2) by initiating treatment at the time of lowest progesterone secretion, i.e., during late metastrum; (3) by initiating estrogen treatment before the onset of reproductive maturity, i.e., at birth and up to a few days of life; and (4) by masculinizing the pituitary gland of the newborn female by androgens followed later, e.g., at day 50 of life, by estrogen treatment.

Induction of tumors may be achieved by subcutaneous implantation or by injection of estrogens. Kirkman and Bacon (1952b) found that the minimum effective total dose was 54 mg given by injection over 180 days or, by implanted pellets, a total dose of 16 or 17 mg over 270 days. The minimum daily dose of diethylstilbestrol (DES) was between 0.03 mg and 0.09 mg per day, and, although 0.6 mg every second day was effective, it ceased to be so if given every tenth day. The latter effect was probably due to an inadequate total amount of stilbestrol administered rather than to discontinuity in absorption.

Kirkman (1959) suggested that, provided the minimum dosage was given, increased dosage did not have significant effect on the latent period or incidence of tumor formation. However, Horning (1956a) found that, by implanting a second pellet of DES 12 weeks after the initial implant, incidence of renal tumors could be raised from 70–80% to 100%. The most potent estrogens with regard to renal carcinogenesis are DES and estradiol. Others, such as estrone, ethynylestradiol, estriol, and Fenocyclin are less effective and have longer latent periods (Kirkman, 1959).

2. *Morphology, Histogenesis, and Ultrastructure*

Most observers agree about the macroscopic appearance of the tumor. After about 200 days of treatment, early stages of primary tumor may be seen on the renal surface and, on section, in the substance as small, pale, solid areas. Distribution is cortical with a tendency for greater

incidence near the corticomedullary border adjacent to the arcuate vessels. As treatment continues, the lesions increase in number with variation in tumor size—possibly indicating different latent periods for nodules within the same kidney—and eventually form multiple, large bilateral tumors, often with hemorrhagic, necrotic, and cystic change (Kirkman, 1959; Bloom *et al.*, 1963a). According to Kirkman and Robbins (1959) the left kidney is usually more involved and sometimes may achieve a weight of 14 mg, which is 23 times greater than normal. Tumors are easily palpable after 9 months of growth (Bloom *et al.*, 1963a).

After 9–10 months of treatment with stilbestrol, the renal tumor is likely to have extended beyond the primary site and, by expansive growth and peritoneal sowing, to have involved contiguous structures. Most frequent sites are the peritoneal surfaces of the diaphragm, the splenic hilus, omentum, and mesentery, parietal peritoneum, the hepatic porta, and the scrotal sac (Kirkman and Robbins, 1959; Bloom *et al.*, 1963a). Secondary deposits on the serosa of the liver and gastrointestinal tract are less frequent, but invasion of the spleen occurs frequently. Occasionally, in long-treated animals, blood- or lymph-borne metastases may be recognized in the lungs and cervical nodes (Kirkman and Robbins, 1959), and sometimes the tumor infiltrates the blood vessels and muscular wall of the ureter (Pol'kina, 1959).

With some reservations, the general consensus of opinion is that the tumors are epithelial in origin, arising from proximal or distal convoluted tubules (Horning and Whittick, 1954; Mannweiler and Bernhard, 1957; Guthrie, 1960; Algard, 1960; Bloom *et al.*, 1963a). Dontenwill (1959) agreed that some tumors were of epithelial origin but that others were of a sarcomatous nature; Kirkman and Robbins (1959), after an extensive review of the literature, concluded that, although by most orthodox morphological criteria the renal tumor was a carcinoma, there was sufficient evidence to suggest a nonepithelial contribution.

Microscopically, multiple, nonencapsulated foci in the cortical area show evidence of tubular cell proliferation with obliteration of the lumina. By local infiltration into surrounding parenchyma, compact masses of pale-staining, round cells are produced. Abundant intracytoplasmic doubly refractile lipoid material is sometimes present in tumor cells. Occasionally, the tumor takes a tubular form, but a papillary or pseudoglandular appearance is more common (Bloom *et al.*, 1963a). Kirkman and Robbins (1959) noted two distinct cell types insofar as a continuous mass of relatively small, deeply staining tumor cells were interrupted by irregularly distributed, discontinuous masses of larger, lightly staining and more basophilic cells. The authors suggested that

the former cells may have been of stromal or vascular origin. Pol'kina (1959) also remarked on similar types of cell.

Mannweiler and Bernhard (1957) examined renal tumors by electron microscopy and found, in addition to various degrees of structural differentiation, the presence of a highly differentiated, ciliated border on tumor cells. The cilium was identical with that seen on protozoa and on ciliated epithelial cells of other species and had not been observed in the kidneys either of control hamsters or of other species. Llombart and Peydro (1970) studied the kidneys of 15 animals treated for 9 months by DES and reported the presence of cilia in the epithelial and mesenchymal parts of the tumor as well as in the tubular epithelium. They considered the tumors to be metanephral derivatives and to be hormone-dependent, differentiated nephroblastomas.

3. *Histochemistry*

The tumors displayed sudanophilic droplets (Kirkman and Robbins, 1959) and neutral lipids (Horning and Whittick, 1954). Tumor cells contained cholesterides with some free cholesterol, chiefly at the periphery of the tumor masses. The plasmal reaction and response to the Ashbel–Seligman test for active carbonyl groups were negative. By the Sakaguchi test, the cells were negative for arginine (Horning and Whittick, 1954), but, by the use of the Thomas test, Kirkman and Robbins (1959) showed that there was a slightly greater concentration of arginine in neoplastic cells than in normal tubular cells. The Millon reaction for tyrosine and the ninhydrin–Schiff method for protein-bound amino groups were negative, but the tumor cells were positive for ascorbic acid and were not metachromatic (Horning and Whittick, 1954; Kirkman and Robbins, 1959). The tumor had a high content of alkaline phosphatase but less acid phosphatase (Kirkman and Robbins, 1959; Dontenwill and Eder, 1957). Glycogen was not demonstrable in the tumor cells (Horning and Whittick, 1954; Kirkman and Robbins, 1959), but, according to Arcadi (1963), glycogen was present but seemed to be in cells of connective tissue origin. Manning *et al.* (1963) demonstrated that there was no detectable change in the dehydrogenases of the Krebs cycle or the hexose monophosphate shunt. Similarly, there was no alteration in lactic and glutamic dehydrogenases. Manning *et al.* (1964) found no quantitative or distributive change in renal β-glucuronidase in estrogenized hamsters. McGregor *et al.* (1960) induced renal tumors in hamsters and studied total fat, protein, carbohydrate, and nucleic acid levels in liver and kidney at 3 months, as tumors became detectable microscopically (8 months), and when lesions were well established (10 months).

Regression after withdrawal of stilbestrol was followed. Changes in the parameters studied occurred only when tumors were well established. Renal function did not change during 8 months of DES treatment with regard to urea clearance, pH, specific gravity, or urinary volume.

Dodge (1973) analyzed isoenzymes of glucose-6-phosphate (G-6-P) dehydrogenase and lactate dehydrogenase in estrogen-dependent and estrogen-independent tumors. The author found that lactate dehydrogenase isoenzyme analysis was of little value in differentiating between hormone-dependent primary renal tumors and hormone-independent or spontaneous renal tumors, nor did it distinguish surrounding kidney tissue from the primary tumors. However, G-6-P dehydrogenase analysis could be used to distinguish hormone-dependent from hormone-independent tumors.

Easty and Ambrose (1955) reported that tumor proteins were characterized by a more random structure, but that there was no significant difference in the overall amino acid composition as compared with normal renal tissue. Ghaleb (1961) found that tritium-labeled DES was taken up by renal tubular epithelium and that the concentration was greater in males than in females—obviously a point of interest in view of the male susceptibility to estrogen-induced tumors. However, Kirkman (1958) demonstrated that tumors could be induced in female kidneys transplanted into male animals, thus indicating that the difference lay in environment rather than in any inherent difference in renal tissue from each sex.

4. *Pituitary Changes*

Vasquez-Lopez (1944) and Koneff *et al.* (1946) studied the effects of chronic administration of DES on the hamster pituitary and agreed substantially in their findings. The main effect occurred in the intermediate lobes and consisted of hypertrophy and hyperplasia to an extreme degree with infiltration of the intermediate cells into the posterior lobe, the infundibulum, and, according to Vasquez-Lopez (1944), the base of the brain. Koneff *et al.* (1946) noted that the invading cells of the pars intermedia were in many respects quite normal but inclined to be more irregular in shape, larger, and more basophilic in staining reaction. There was enlargement of the Golgi apparatus. Numerous vesicles, often large and irregular in outline and filled with basophilic hyaline material, were commonly present. The destruction of cells of the intermedia and their transformation into "Herring bodies" was apparently accelerated by estrogenization. Vasquez-Lopez (1944) did not find increased mitotic activity, although Koneff *et al.* (1946) found that to be a feature in their animals. The same authors described increased

number and activity of acidophile cells in the anterior lobe of treated male animals associated with a decreased chromophobe population. Hamilton *et al.* (1974) reported an increase in the prolactin cells of the anterior lobe as well as changes similar to those described by Koneff *et al.* (1946) in the intermediate lobes.

Kirkman and Bacon (1948) and Kirkman (1959) described massive hypophysial adenomas which invaded the hypothalamus in DES-treated hamsters. Horning and Whittick (1954) found hypophysial tumors in 28 of 40 similarly treated animals, the majority of which were chromophobe adenomas with a smaller number arising from the intermediate lobe, and Horning (1956) recorded involvement mainly of the pars intermedia.

Dontenwill and Eder (1957) described adenomatous lesions of the pituitary, and Dontenwill (1959) further reported that, in one-third of all treated animals, large pituitary adenomas were present and contained granulated basophiles, amphophiles, and Crooke cells. Russfield (1963) noted chromophobe adenomas of the anterior and tumors of the intermediate lobes in hamsters under prolonged estrogen treatment. Six tumors, average weight 34.5 mg, of pars intermedia occurred in castrated animals; a further six tumors in intact males achieved an average weight of 53.9 mg. One of the latter tumors arose in the intermedia, and the remaining five were chromophobe adenomas with associated intermediate lobe hyperplasia. Animals with chromophobe adenomas also had adrenal changes suggestive of early neoplasia. The author hypothesized that the differences between the pituitary changes in intact and castrated males may have been linked with the adrenal changes.

5. *Hormone Dependency and Regression*

The withdrawal of estrogen for at least 115 days from primary renal tumor-bearing hamsters—intact males or gonadectomized males or females—causes regression of the tumors. There is a decrease in size, the color changes from white to amber, the lesions are transformed into vesicles filled with clear, colorless fluid, and, finally, liquefaction occurs (Kirkman, 1959). The same author stressed the point that regression should not be confused with viability since, even after a regression period of 200 days, administration of estrogen caused regrowth of the tumors within 57 days. Additionally, transplantation of regressed tumor-remnants into a new host often resulted in tumor formation. Kirkman (1959) also noted that "redifferentiation" may occur in regressing tumors with the formation of tubular structures and suggested that this process was related to the presence of undifferentiated metanephrogenic blastema in the hamster kidney. It was of note that regression had not

occurred by 50 days after hypophysectomy of tumor-bearing animals. McGregor *et al.* (1960) treated animals for 10 months with stilbestrol, and 1 month after withdrawal of the estrogen found complete regression of the tumors in only 60% of the animals.

6. *Factors Influencing Induction and Growth Rate of Primary Tumors*

a. Hormones and Chemicals. i. *Testosterone.* Horning (1956b) implanted hamsters with a pellet of DES and treated them with testosterone propionate in doses up to 2–5 mg per week, thereby inhibiting the induction of renal tumors. Kirkman (1951, 1959) produced a similar effect by implanting pellets of testosterone propionate. However, the same author showed that implantation of 30-mg pellets of testosterone did not cause regression of established tumors but, on the contrary, encouraged metastatic growth.

ii. *Progesterone.* Kirkman (1951, 1959) and Kirkman and Wurster (1957) found that concurrent implantation with stilbestrol and progesterone pellets inhibited the induction of renal tumors in intact and castrated males and in ovariectomized females.

iii. *Corticosteroids.* Implants of deoxycorticosterone concurrently with DES inhibited renal tumor induction (Kirkman, 1959; Rivière *et al.*, 1960). However, cortisone combined with stilbestrol increased the incidence of primary renal tumors and of metastases in animals bearing renal tumor implants (Kirkman, 1959).

iv. *Prolactin.* Ovine prolactin administered to intact male hamsters did not induce renal tumors. When given concurrently with DES, there was no obvious difference in induction period or in the number or severity of the tumors when compared to those of animals given DES alone (Hamilton *et al.*, unpublished data).

v. *Chemicals.* Injection of 2-Br-α-ergokryptine-methanesulfonate (CB 154) along with DES resulted in a marked reduction in the incidence and severity of renal tumors in intact males as compared with animals treated only with DES (Hamilton *et al.*, 1974). Kirkman and Horning (1957) found that implantation of 20-methylcholanthrene in DES-treated animals similarly reduced the incidence and the growth rate of the resulting renal tumors.

b. Effects of Unilateral Nephrectomy or Ureterectomy. Horning (1955) performed unilateral nephrectomy on hamsters and followed this 4–5 or 10–12 weeks later by the implantation of 20-mg pellets of pure DES. The author found that nephrectomy had accelerated the appearance of renal carcinomas with mean latent periods of 190 ± 28.7 and 286 ± 23.5 days for the DES-treated nephrectomized group and DES-

treated animals, respectively. Additionally, it was observed that large tumors arose quickly in any kidney remnants left from the nephrectomy. Horning suggested that, although the liver was the main organ for inactivation of estrogen, the kidney also performed some of this function, and, consequently, the remaining kidney accumulated a greater concentration of estrogen with resultant neoplasia. Bloom *et al.* (1963a) thought that a more likely explanation was the fact that a kidney stimulated to hypertrophy was more sensitive to the effects of a carcinogen. However, according to Kirkman (1959) compensatory renal hypertrophy does not occur in the estrogen-treated hamster.

Ising (1956) unilaterally ureterectomized male hamsters and implanted DES pellets. The animals developed a higher incidence of renal tumors, which were consistently and markedly larger on the operated side as compared with DES-treated control animals. Ising ascribed this effect to either an increased concentration of estrogen in the ureterectomized kidney or increased susceptibility of the damaged organ to the effect of carcinogen. Kirkman (1959) and Robbins (1959) failed to confirm the work of Horning (1955) and Ising (1956), respectively. Nevertheless, the effect of renal trauma on the induction of tumors cannot be ignored entirely. From the results of an additional experiment in which one kidney of each of 11 males was traumatized by means of needles prior to stilbestrol implantation, Kirkman (1958) concluded that mechanical traumatization predisposed renal cortical tissue to neoplastic transformation.

7. *Transplantation*

Kirkman (1951, 1959) and Horning (1956a,b) showed that the estrogen-induced renal tumor could be transplanted and carried serially in intact or gonadectomized hamsters of either sex, provided that the recipient had been pretreated with estrogen before grafting. Initially, a latent period of 3–7 months (Horning, 1956a), 100 days (Kirkman, 1959), or, unusually, 2 weeks (Dontenwill and Eder, 1957) elapsed before the subcutaneous implants became palpable. With repeated transfer, the success rate increased and the latent period decreases until by the twenty-third transfer the tumor was palpable at 1 month and the shortest observed latent period for metastases from a transplant was 59 days (Kirkman, 1959). Kirkman and Horning (1957) reported successful takes in untreated male hamsters, so that the tumor, after nearly 5 years of serial grafting, eventually ceased to be dependent on administration of exogenous estrogen. However, the tumors became palpable only after an average period of 11.5 months. With each succeeding generation, that period steadily decreased, until Bloom *et al.* (1963a) reported that,

after 9 years of repeated transfer, tumors of the forty-fifth generation became palpable in the flank of practically every animal within 2–3 weeks of transplantation. Success varied with the site of implantation. Whereas intrathoracic implants gave poor results, cheek pouch and subcutaneous implants did well with almost 100% success rate (Horning, 1956b; Dontenwill and Eder, 1957; Kirkman, 1959). Horning (1956a) found, additionally, that when tumors were grafted into the subcutaneous tissues of the tail region there was a higher number of blood-borne metastases than if implants were inserted into the subcutaneous tissue of the trunk.

Transplant growth may be altered by the administration of various hormones. Testosterone propionate increased the rate of growth by approximately twice that resulting from estrogen alone, whereas with deoxycorticosterone acetate or progesterone, growth was decreased. If tumors were grafted into animals along with 30-mg pellets of testosterone, the implant did not grow, thus indicating that testosterone could not substitute for estrogen in the establishment and further growth of a first serial passage of the renal tumor (Kirkman, 1959). The fact that testosterone stimulated transplant growth led Horning (1955) to suggest that the androgen may have been converted to estrogen by various organs, although Kirkman (1959) disagreed since he had failed to induce primary tumors after long-term treatment of hamsters with testosterone. Dontenwill and Ranz (1960) gave growth hormone to castrated male animals with implanted tumors. Despite a great increase in body weight in those animals (78%) as opposed to controls (13%), the tumors failed to grow, thus underlining the fact that a strong growth stimulator was not sufficient for tumor growth. Cortisone increased the incidence of primary renal tumors and of metastases in stilbestrol-treated tumor transplant-bearing animals (Kirkman, 1959).

Bloom *et al.* (1963a) used estrogen-independent renal tumor implants to study the effect of hormones on growth. Provera (6α-methyl-17α-hydroxyprogesterone), cortisone, and testosterone were given subcutaneously at a dose of 2.5 mg twice weekly, 2.5 mg 5 times per week, and 2.5 mg twice weekly, respectively. Testosterone or Provera treatment induced active proliferation of the implants whereas in animals treated with cortisone alone or in combination with Provera, the tumors showed extensive areas of necrosis and hemorrhage. In adjacent areas, reduced mitotic activity or varying degrees of tumor-cell degeneration accompanied by an inflammatory reaction were seen. No loss of inhibitory action of cortisone was recorded over 5 serial transplantations of the tumor. In a later report Bloom (1964) indicated that Provera reduced the growth rate of transplanted estrogen-dependent tumors and that

inhibition of the transplanted estrogen-independent tumor was finally achieved by markedly increasing the dose of Provera.

Bloom *et al.* (1963b) continued investigation of the growth of estrogen-independent tumor transplants and assessed the influence of endocrine ablation. The effect of bilateral adrenalectomy, castration, and a combination of both were studied. In all adrenalectomized animals there was evidence of tumor inhibition, castration having a greater inhibitory effect. Castration of animals, already adrenalectomized and with tumor growth, led to actual tumor regression.

In further experiments, the effect of castration on established tumors was ascertained. Immediately after castration there was a marked reduction in tumor growth rate and, until the experiment was terminated at day 93, little change in size took place. A final experiment revealed that treatment with estradiol monobenzoate completely neutralized the inhibitory effect of orchiectomy. Those findings suggested that, although the transplanted tumor was said to be estrogen-independent, it required intrinsic estrogen derived from the testes, and possibly the adrenal, for growth. In a later publication, Bloom *et al.* (1967) assessed the effect of an estrogen antagonist, U 11,100 A, 1-{2-[*P*-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl) phenoxy]ethyl}pyrollidine hydrochloride, on the estrogen—independent tumor implant. The chemical at a dose of 1 mg per day for 6 days per week inhibited tumor growth, and the average survival time of treated animals extended to 86 days compared with only 19 days for untreated controls. Simultaneous administration of a daily dose of 0.25 mg of estradiol benzoate abolished the inhibitory effect of U 11,100 A.

8. *Tissue Culture and Antigenic Aspects*

Algard (1960) showed that cells from an estrogen-dependent renal tumor could be grown in hormone-free media and that the addition of crystalline hormone did not enhance growth or survival. However, in organ culture, growth did not occur and survival time was greatly reduced unless estrogen was added to the media. Because of the *in vitro* hormone dependence, the author considered that induction and maintenance of the tumor *in vivo* was related to a direct effect of estrogen on the kidney. Algard (1963) further reported that, when cultures of renal tumors were implanted into animals, growth occurred only in the presence of exogenous estrogen.

Dontenwill and Wrba (1959) examined organ cultures of the renal tumor and found that after 48 hours there was separation into fibroblasts and a central core of tumor cells. The addition of normal hamster serum caused necrosis of the tumor cells whereas, when estrone sulfate was