Progr. Exp. Tumor Res. *14:* 59–88

Inhibition of Tumor Induction in Chemical Carcinogenesis in the Mammary Gland[1]

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Contents

1 This study was supported by US Public Health Service grant CA-04632.

I. Introduction

The idea that carcinogenesis involves 2 separate components with independent mechanisms arose out of observations made by ROUS and KIDD [1941] in their study of the regression of skin tumors in the rabbit, and by BERENBLUM [1941] in his study of the action of the croton oil in the induction of skin tumors. This observation led to the postulate that limited treatment with a carcinogen caused conversion of a few epithelial cells into 'latent tumor cells' which remained in a dormant state and required further stimulation to develop into a tumor. A '2-stage' mechanism was thus conceived: the process of 'initiation' triggered by a carcinogen, and a succeeding process of 'promotion' induced by 1 or more agents enhancing the development of a visible tumor from 'latent tumor cells.' It is suggested that the promoting agent need not be a carcinogen.

The significance of the concept of a multistage mechanism rests not on the exact number of stages involved but, rather, on the implication that the process of carcinogenesis includes more than one component. The components may obviously act either independently or in concert. Some experiments reported earlier by the present author [DAO, 1962a, b] clearly suggested that in carcinogenesis in the mammary gland, steroid hormones act not only as co-carcinogens or promoting agents, but may also be indispensable for the initiation process.

The earlier studies by ROUS and by BERENBLUM also led to the conclusion that 'initiation' is an irreversible process, whereas promotion can be blocked, thus inhibiting appearance of the tumor. The hypothesis that the initiating process is instantaneous and irreversible, however, is debatable. The difficulty of such a postulate arises when there is a lack of delineation between initiation and promotion. Nonetheless, evidence is overwhelming that somewhere along the path, completion of carcinogenesis can be interfered with, i.e., appearance of a visible tumor can be prevented. Studies of inhibition of carcinogenesis have important and significant implications: (1) they may lead to better understanding of the mechanism of carcino-

genesis, and (2) conceivably, methods for inhibition of tumorigenesis in humans can be derived from experimental studies.

Inhibition of carcinogenesis has been investigated in several target tissues, including the skin, liver, and mammary gland. This paper is concerned primarily with the inhibition of mammary-gland carcinogenesis induced by chemical carcinogens. We will consider mainly inhibition of mammary tumor induction prior to the appearance of visible tumors. Although it appears that a brief review of the factors associated with the process of carcinogenesis in the mammary gland is necessary for consideration of the inhibition of tumor induction, it is a difficult task to condense the mass of data into a few pages. The author suggests that readers refer to a recent review on this subject [DAO, 1969a].

It can be said with certainty that if we understood the mechanism of chemical carcinogenesis, we would be able to develop methods to inhibit the induction of tumors by carcinogens in the target tissue. Although exact knowledge pertaining to the mechanism of carcinogenesis is lacking, some major events occurring during the process of carcinogenesis have been studied extensively. It is on the basis of this body of knowledge that investigations have been carried out to gain insight into the relationships between the induction of tumors by chemical carcinogens and the inhibition of carcinogenesis by various mechanisms.

II. Inhibition of Mammary Tumor Induction by Hormonal Stimulation of the Target Tissue

A. Inhibitory Effects of Pregnancy and Lactation in Carcinogenesis in the Mammary Gland

The hormonal aspects of tumor induction and growth in rats treated with polycyclic aromatic hydrocarbons have been studied in several laboratories. One of the interesting earlier observations from these studies is the finding that hormones that can inhibit induction of mammary tumors in rats simultaneously induce exuberant growth of the normal mammary gland, but under different circumstances these hormones greatly augment tumor growth and accelerate tumor induction.

DAO and SUNDERLAND [1959] observed that pregnancy and pseudopregnancy markedly decreased the latent period of mammary cancer in rats fed multiple doses of 3-methylcholanthrene (3MC). These authors

showed that although the tumor incidence in the control group fed with 10 mg of 3MC a day for 20 days was 45%, pregnancy (rats mated 4 days after the last dose of 3MC) caused a significant increase in tumor incidence (to 89%) in rats that received an equivalent amount of carcinogen. It was also shown that a decreased mammary tumor incidence caused by reduction in 3MC doses could be restored to a significantly higher tumor incidence if pregnancy occurred after carcinogen treatment. Interestingly, the latent period for tumor appearance was practically the same in pregnancy groups, irrespective of the total dose of carcinogen given. These authors also discovered, however, that when identical doses of 3MC were given to the already pregnant rats, mammary tumors failed to develop in these rats. Similar experiments were done with *postpartum* lactating rats, and the results were similar. In these instances, pregnancy and lactation obviously inhibited tumor induction.

Subsequent studies by DAO, BOCK and GREINER [1960] not only confirmed the earlier observations but also demonstrated the critical significance of time between carcinogen administration and mating of the carcinogen-treated females. Table I shows that the incidence of mammary tumors in rats fed a single dose of 3MC increases as the interval between the time of carcinogen administration and mating (and pregnancy) lengthens. The data show that if female rats are mated immediately after carcin-

Table I. Influence of pregnancy on incidence of mammary tumors in rats[1]

Group	Interval[2] (days)	Number of rats	Rats with tumors		Appearance of palpable tumors (days)		
			Number	Percent	Range	Median	Mean
Control	—	20	12	60	50–137	125	110
Pregnancy	1	20	1	0.5	40	40	40
Pregnancy	10	32	3	9	30–45	45	40
Pregnancy	15	32	4	12	33–45	40	39
Pregnancy	20	29	7	24	35–44	40	39
Pregnancy	25	23	8	35	37–45	39	40
Pregnancy	30	10	5	50	35–47	42	42

1 All animals received a single feeding of 30 mg of 3MC in 1 ml sesame oil, and were observed for 5 months.

2 Interval between feeding 3MC and mating.

ogen treatment, mammary tumors fail to develop. This experiment suggests that there is a critical time period between administration of carcinogen (or localization of carcinogen in the cells) and transformation of previously normal cells into 'latent tumor cells.' It seems that tumor induction can be inhibited during this period. If such is the case, then the initiation of carcinogenesis, in the mammary gland at least, can be neither instantaneous nor irreversible.

In their earlier experiments, DAO and SUNDERLAND [1959] also found a rapid regression of mammary tumors after parturition, but if rats became pregnant again, a rapid progression of tumors was observed. The rapid regression of tumors following parturition is due to the sudden removal of hormones derived from the placenta and the decreased activity of the hosts' own gonads and adrenals rather than a 'direct effect' of lactation. DAO, BOCK and GREINER [1960], however, showed that if a carcinogen was administered to *postpartum* rats during lactation, induction of mammary tumors was completely inhibited.

The inhibitory effect of pregnancy and lactation on induction of mammary cancer cannot be fully explained. Several factors should be considered: (1) it is possible that large quantities of hormones secreted by the pituitary, gonads, adrenals, and placenta can destroy cancer in its incipient stage in the host; (2) localization of this carcinogen and the rate of metabolic clearance in the target tissue are altered and interefered with by pregnancy; and (3) lactation induces a direct local effect of suckling, which may be inhibitory to tumor induction.

Studies of localization and clearance of the carcinogen 3MC were compared in host tissues of virgin, pregnant, and lactating rats [DAO, BOCK and GREINER, 1960]. Although there was no significant difference in the concentration of 3MC in the mammary gland in virgin and pregnant females, the rate of clearance of the hydrocarbon appeared to be much more rapid for the mammary gland in the pregnant rats. In the lactating rats, furthermore, the 3MC reached only a low level of concentration in the mammary gland (6–7 μg/gm of tissue within 24 h as compared with 22–25 μg/gm of tissue in virgin females). The rate of clearance of 3MC from the mammary gland was rapid; the carcinogen was rapidly excreted in the milk, as was shown by the high concentration of 3MC in the milk curd of the suckling newborn.

That the inhibitory effect of lactation was not due to a 'direct local effect of suckling' was clearly demonstrated by experiments in which fetuses were removed by cesarean section before parturition [DAO, BOCK and

GREINER, 1960]. In these experiments, the tumor incidence in the group in which cesarean sections were done was the same as that in the rats in which full lactation and suckling were permitted after parturition. If the inhibition of tumor appearance was due to a direct local effect of suckling, then the incidence of mammary tumors in rats that have never lactated should be the same as that in controls. This clearly is not the case.

B. Inhibition of Tumor Induction by 17β-Estradiol and Progesterone

HUGGINS, MOON and MORII [1962] described 2 methods that were effective in the inhibition of induction of mammary tumors in rats receiving DMBA: (1) administration of 17β-estradiol together with progesterone 15 days after carcinogen treatment, and (2) stimulation of the ovary with equine gonadotrophin to increase ovarian steroid hormone production. These authors reported that concurrent injection of 20 μg of 17β-estradiol and 4 mg of progesterone each day for 30 days beginning at 15 days following a single feeding of DMBA resulted in a total inhibition of mammary cancer induction in 52% of the rats so treated. Of the 52 rats free of mammary cancer, however, 31% developed mammary fibroadenoma later. Progesterone alone induces proliferation of mammary epithelium, consisting of hyperplasia and marked increase in the alkaline phosphatase of the mammary gland, and accelerates the growth rate of mammary cancer; 17β-estradiol in a small dose (0.1–1.0 μg) exerts little effect on the growth rate of mammary cancer, but induces constant estrus, however. Reduction in tumor incidence and prolongation of the latent period are the results when larger doses, 10–20 μg, are used. In these rats, the mammary glands are hyperplastic and contain milk secretion. Concurrent administration of progesterone and 17β-estradiol induces a profound gestational effect in the mammary gland, consisting of hyperplasia of mammary tubules but with minimal secretion in the acini—an exaggerated progesterone effect.

HUGGINS and YANG [1962] suggested that concurrent administration of progesterone and 17β-estradiol was able to destroy the cancer at its incipient stage. Similar results were obtained by injection of 1 U of equine gonadotrophin for 10 days, which induced marked gestational changes in the mammary gland but inhibited mammary cancer induction in 30% of the rats. It was suggested that the ovary can be stimulated to produce sufficient quantities of its own hormones to destroy cancer in the host.

C. Inhibition of Tumor Induction by Lesions of the Hypothalamus

CLEMENS, WELSCH and MEITES [1968] showed that placement of lesions in the median eminence in rats prior to a single i.v. dose of 5 mg of DMBA significantly inhibited the induction of mammary cancer by the carcinogen. In the intact control group, 95% of the rats developed mammary cancer, whereas intact rats with bilateral lesions in the median eminence had only a 30% incidence of mammary tumors. Induction of mammary tumors was completely inhibited if rats with bilateral lesions in the median eminence were also castrated. These authors also showed that if lesions of the median eminence were placed 75 days after DMBA administration, a marked stimulation of growth of mammary cancer occurred. These observations were subsequently confirmed by others [KLAIBER, GRUENSTEIN, MERANZE and SHIMKIN, 1969].

It is well established that placement of lesions in the median eminence of the tuber cinereum causes an enhanced release of prolactin and a marked reduction in the release of all other anterior pituitary hormones [MEITES, NICOLL and TALWALKER, 1963]. Prolactin induced a marked stimulation of mammary growth, but rendered the mammary gland refractory to DMBA injury and inhibited mammary tumor induction.

D. Inhibition of Tumor Induction by Multiple Pituitary Grafts and Progesterone

WELSCH, CLEMENS and MEITES [1968] investigated the effects of prolactin-secreting pituitary homografts and progesterone on the induction of mammary tumors by DMBA. Five groups of rats were studied: (1) intact controls (2) ovariectomized, (3) intact with 4 pituitary homografts, (4) ovariectomized with 4 pituitary homografts, and (5) rats receiving 4 mg of progesterone a day. All of these procedures were done about 30 days before DMBA treatment. The results show that ovariectomy with or without four pituitary homografts completely inhibited the induction of tumors. Four pituitary grafts or progesterone in intact rats also inhibited mammary tumor induction, but to a much lesser extent. These experiments illustrate inhibitory effects on tumor induction as the result of overstimulation of the target tissue or of depletion of the hormone essential for initiation of carcinogenesis (to be discussed later).

E. Discussion

The experiments described [DAO, BOCK and GREINER, 1960; HUGGINS, MOON and MORII, 1962; CLEMENS, WELSCH and MEITES, 1968] have certain similarities; but whether the mechanism by which tumor induction is inhibited is the same in all 3 studies is not entirely clear. We shall examine several possibilities.

The experiments in which complete inhibition of tumor induction was made possible if carcinogen was given to the already pregnant rats clearly demonstrates that hyperfunctioning mammary epithelial cells are refractory to carcinogenic stimuli. This gestational effect caused by pregnancy can also be observed in the mammary glands of male rats of the same strain. The mammary glands of mature male rats are always in a state of hyperplasia, and they are resistant to carcinogenesis induced by aromatic hydrocarbons. Testicular hormones stimulate mammary growth in male rats and protect the mammary gland from injury by carcinogenic hydrocarbons, thus inhibiting carcinogenesis. Removal of testicular hormones by castration and introduction of estrogens by ovarian grafts are conducive to induction of mammary cancer in the male host [DAO and GREINER, 1961]. The experiments of CLEMENS, WELSCH and MEITES [1968] clearly are similar to those of DAO, BOCK and GREINER [1960] in that the increased release of prolactin induces hyperplastic changes in the mammary gland. CLEMENS, WELSCH, and MEITES [1968] observed that transplantation of pituitaries into intact rats prior to DMBA treatment similarly inhibits mammary tumorigenesis, and at the same time stimulates mammary growth. In the intact rats, increased prolactin release as a result of lesions of the median eminence may stimulate progesterone secretion by the ovaries, and hence mammary hyperplasia. These experiments of tumor inhibition have one common denominator, i.e., a hyperfunctional mammary gland.

Why is a hyperfunctional mammary gland refractory to carcinogenesis? It appears that cells undergoing active division and proliferation are resistant to carcinogenic stimuli and transformation. Both testicular and ovarian hormones are effective in the inhibition of mammary tumor induction, but both induce mammary hyperplasia. It seems reasonable to suggest that the presence of excessive amounts of steroid hormones in the receptor sites of the mammary tubules blocks the concentration of the carcinogenic agents in the mammary gland, and hence the induction of mammary tumors. In these instances, initiation of carcinogenesis has never occurred. Such a hy-

pothesis, however, does not explain why mammary tumor induction can be inhibited when steroids are given 10 or 20 days after the administration of carcinogenic agent, during which time initiation of carcinogenesis must have already taken place [HUGGINS, MOON and MORII, 1962].

In HUGGINS, MOON and MORII's experiments, evidence seems to support their postulate that steroid hormones can indeed destroy mammary tumors by 'direct action'. It seems that progesterone in combination with 17β-estradiol can interfere with the steroid hormone mechanism that is of paramount importance for the growth of some mammary tumors.

In the experiments of DAO and SUNDERLAND [1959], 10 mg of 3MC was given daily for 20 days, and mating began 4 days after the last dose of 3MC. In contrast, the later experiments by DAO, BOCK and GREINER [1960] were carried out in rats given only a single dose of 30 mg of 3MC. This marked difference in carcinogen dosage may be the important factor in inhibiting tumor induction, by actually reducing significantly the carcinogenic stimuli to the cell population, and thus markedly decreasing the incidence of tumors. In this situation, subsequent pregnancy augmented the tumor growth rate, but not the tumor incidence.

The fact that tumor incidence rises as the time interval between 3MC administration and pregnancy increases is strongly indicative of the important relationship between the dose of carcinogen and the time required for induction. A weaker carcinogen or a 'subcarcinogenic' dose requires a longer time for initiation of carcinogenesis. The promoting factor, in this case, pregnancy, can accelerate tumor growth but not tumor incidence.

The mechanism by which lactation inhibits mammary tumor induction is distinctly different. The inability of the lactating glands to concentrate a carcinogen because of the rapid excretion of the carcinogen in the milk prevents localization of the carcinogen in the mammary gland, a *sine qua non* to carcinogenesis.

III. Inhibition of Mammary Tumor Induction by Hormonal Deprivation: Evidence for Hormone Regulation of Interaction Between Carcinogen and Target Tissue

A. Inhibition of Mammary Tumor Induction by Castration: A Time Factor

In carcinogenesis in the mammary gland, it has been repeatedly demonstrated that administration of potent carcinogens such as 3MC or

DMBA to rats without ovaries or hypophysis is less effective in the induction of mammary tumors [HUGGINS, BRIZIARELLI and SUTTON, 1959; DAO and SUNDERLAND, 1959]. The absence of hormones from the ovaries and hypophysis markedly decreased the incidence of tumors but did not prevent tumor formation. In the experiments of HUGGINS, BRIZIARELLI and SUTTON [1959], simultaneous injections of either 17β-estradiol or progesterone and feeding of 3MC in ovariectomized rats restored the incidence of tumors to the control level. The failure of mammary tumors to develop in these experiments thus appears to be due to a lack of the promoting effects of the hormones. The question whether 3MC or DMBA can initiate the induction of mammary carcinogenesis cannot be answered by these experiments.

To answer this question, we removed the ovarian hormones by castration at the time of carcinogen administration, and a pair of ovaries was grafted into these rats 20–25 days later. It is postulated that if 3MC or DMBA can induce the initiation of carcinogenesis in the mammary gland in the absence of estrogen, mammary tumors should develop from the 'latent tumor cells' when ovarian hormones are supplied by the functioning ovarian grafts.

Two sets of experiments were carried out [DAO, 1962 a, b].

(a) Excision of ovaries after carcinogen treatment. In this study, ovaries were removed from the rats at various intervals after a single feeding of 20 mg of DMBA. The results show that if castration was performed 24 h after oral administration of DMBA, induction of mammary tumor was profoundly inhibited, since grafting of a pair of ovaries later failed to induce the development of tumors. Removal of ovaries more than 7 days after DMBA treatment, however, did not prevent or reduce the development of mammary tumors, since tumors appeared when ovaries were grafted into the animals. These experiments strongly suggest that neoplastic transformation of the mammary gland fails to occur if the ovaries are removed shortly after the administration of DMBA. On the other hand, if the ovaries are removed after adequate exposure of the mammary gland to DMBA, initiation has already occurred. The 'latent' tumor cells grow into a visible tumor when promoting hormones are supplied by the functioning grafts.

(b) Excision of ovaries before carcinogen treatment. In this experiment, 20 mg of the carcinogenic hydrocarbon DMBA was fed to female rats at various times (7, 15 and 30 days after castration), and ovaries were transplanted on the same day of DMBA feeding, or 7, 15 or 30 days after

DMBA feeding. The mammary tumor incidences were similar in groups in which DMBA was given 7 and 15 days after castration and ovaries were transplanted on days 0 and 7 after DMBA feeding. The tumor incidence, however, was much lower if ovaries were transplanted 15 or 30 days after DMBA in these 2 groups. The results suggest that restoration of ovarian function in castrated rats in the initiation phase increases tumor induction. In contrast, if the carcinogenic hydrocarbon was given 30 days after castration, induction of mammary tumors was completely inhibited, since no tumors developed after pairs of ovarian grafts were implanted 0, 7, 15, or 30 days after administration of the hydrocarbon. If latent tumor cells were present, tumors should have appeared in the presence of functioning ovaries. The results were negative.

Two significant conclusions can be drawn from this experiment: (a) in rats deprived of ovarian hormones, the incidence of mammary tumors in DMBA-treated rats decreased as the interval between castration and carcinogen administration lengthened; and (b) the interval between DMBA feeding and subsequent ovarian grafting may influence the mammary tumor incidence in these castrates.

B. Inhibition of Induction of Mammary Tumors in Male Rats

Mammary glands of male rats are refractory to carcinogenesis induced by polycyclic aromatic hydrocarbons. We (Dao and Greiner, 1961] demonstrated that induction of mammary cancer was inhibited irrespective of the amount of carcinogen given. Even so, when the carcinogen was given to castrated male rats bearing ovarian grafts, mammary tumor incidence rose significantly. In male rats, a dose of 30 mg of 3MC given in a single feeding failed to induce any mammary cancer. Castrated male rats bearing functional ovarian grafts and receiving a single feeding of 30 mg of 3MC had a tumor incidence of 53%. If, however, the carcinogen was given to the castrated males first, and ovaries were transplanted to these castrated rats 30 days later, the mammary tumor incidence was only 13%. It must be mentioned that the time of introduction of the ovarian hormone is critical. Grafting ovaries into castrated male rats receiving 3MC prior to ovarian transplantation fails to induce mammary cancer [Dao, 1962]. This fact strongly suggests that initiation of mammary carcinogenesis cannot take place in the absence of ovarian hormones.

C. Lack of an Effect of Prolactin in Mammary Tumor Induction

The most conclusive evidence that estrogens are critical in the initiation of mammary cancer in rats is provided by experiments involving male rats. Male rats can develop mammary cancer as often as female rats if they are castrated and implanted with a pair of ovaries. The foregoing experiments have clearly established that conclusion. The pituitary hormone prolactin is critically needed for stimulating and maintaining tumor growth, but it cannot initiate the induction of mammary tumors.

The following experiments illustrate the effects of pituitary grafts on mammary tumorigenesis in both male and female rats:

Pituitary grafts were implanted into castrated male rats that were fed, 2 weeks later, 10 mg of 3MC a day for 15 days. The pituitary grafts stimulated mammary gland growth and, in many cases, lactation; however, there was no increase in tumor incidence [DAO and GREINER, 1961]. Prolactin released by a pituitary graft can perform three functions: mammotrophic, luteotrophic, and lactational. These functions not only can stimulate mammary gland growth, but they likewise promote the growth of mammary tumors. KIM and FURTH [1960], KIM, FURTH and YANNOPOULES [1963] and STERENTAL, DOMINGUEZ, WEISSMAN and PEARSON [1963] showed that mammotrophic hormone, mainly prolactin, was of importance in promoting and maintaining the growth of carcinogen-induced mammary cancer. In our experiments, hormones from the pituitary grafts were effective in stimulating mammary growth [DAO and GAWLAK, 1963], but they did not induce tumor growth.

DAO and GAWLAK [1963] reported profound growth of the alveolar lobules of the mammary gland after induction by pituitary homografts in hypophysectomized or hypophysectomized and ovariectomized rats. DMBA (20 mg) fed to ovariectomized rats bearing functioning pituitary grafts failed to induce any mammary tumors [DAO, 1967].

Lesions of the hypothalamus increase the release of prolactin, but cannot induce mammary tumors in ovariectomized rats [CLEMENS, WELSCH and MEITES, 1968]. In contrast, lesions of the median eminence of the hypothalamus in female rats bearing DMBA-induced mammary cancer stimulate tumor growth [KLAIBER, GRUENSTEIN, MERAUZE and SHIMKIN, 1969]. Using pituitary homografts as the source of prolactin, WELSCH, CLEMENS and MEITES [1968] further confirmed the earlier work of DAO and GREINER [1961], showing that prolactin alone cannot induce the initiation of mammary cancer in rats receiving DMBA.

It is well established that estrogen and prolactin are necessary to maintain mammary tumor growth in rats. Estrogen is believed to act in part by stimulating prolactin secretion [NICOLL and MEITES, 1962]. That estrogen is critically needed in the initiation stage of neoplastic transformation is amply demonstrated by these experiments.

The foregoing experiments also clearly refute the old concept that the role of hormones in mammary carcinogenesis is merely to provide a developed mammary gland on which a carcinogen can act. A well-developed mammary gland is not a requisite for carcinogenesis.

D. Discussion

From the foregoing experiments, it seems clear that DMBA cannot initiate carcinogenesis in the rat mammary gland in the absence of estrogen. In every experiment described, induction of mammary cancer was inhibited when the source of estrogen was removed at the time of DMBA administration. Evidence is overwhelming to suggest that interaction between DMBA and cellular constituents of mammary tissue is interfered with, since these experiments are designed to test whether initiation of neoplastic transformation can occur in estrogen-depleted rats. It is reasonable to conclude that if the initiation of carcinogenesis in the mammary gland can occur without estrogen, mammary tumors should develop when ovarian hormones are supplied by functioning ovarian grafts. This is indeed not the case.

The mechanism by which estrogen regulates the interaction of a carcinogen with cellular constituents is not understood. At best, we can only speculate briefly on how estrogen may control the initiation of carcinogenesis. Two possibilities are suggested:

(a) Estrogen regulates the interaction by modifying the mitotic activity of the mammary epithelial cells. Estrogens arouse mitotic activity in many tissues of the body, this effect being especially pronounced in the gonads and accessory reproductive organs of both female and male. BULLOUGH [1955] has reported that the addition of estrogens to mouse skin incubated in saline containing glucose induces increased mitosis in a 4-hour period. It has been suggested that carcinogenic agents of different kinds, whether radiation or chemical carcinogens, have the common property of interfering with the nucleus of a dividing cell in a characteristic way, and that a cell is most easily affected by carcinogens during mitosis. Although

the effects of estrogen in mitogenic activity in the mammary gland have not been widely studied, work in our laboratory [KOYAMA, SINHA and DAO, 1969] shows that the mitotic index in the mammary glands of female rats decreased gradually from a peak of 1% in 25-day-old rats to 0.1% in rats 100 days old or older. The mitotic index is 0.25% in the mammary gland in 55-day-old rats, an age group that is most susceptible to carcinogenesis induced by polycyclic aromatic hydrocarbons [HUGGINS, GRAND and BRILLANTES, 1961; DAO, 1969].

The data of the foregoing experiments demonstrate that only at a certain rate of mitosis is a cell susceptible to injury by a carcinogen; cells in too rapid or too slow mitosis are not vulnerable to the action of a carcinogen. Perhaps one function of estrogen in this mechanism is the maintenance of a critical rate of mitosis.

The most recent experiments in my laboratory dealing with the effects of local application of DMBA in the mammary gland and the subsequent development of tumors in it [SINHA and DAO, unpublished data] reveal that local 'dusting' of minute quantities of DMBA over the mammary gland induced mammary cancer only in the parts of the gland exposed. The incidence of tumors increased markedly if a small amount of estrogen was 'dusted' over the mammary gland simultaneously with DMBA. The small amount of estrogen did not induce any changes in the endocrine glands. The mammary gland receiving local application of estrogen is not morphologically different from the contralateral gland. The only difference is that mitotic activity in the estrogen-'dusted' gland is accelerated. It appears that the increased mitotic activity of the mammary epithelial cells makes them more vulnerable to attack by a carcinogen. Similar observations have been made by us [KOYAMA, SINHA and DAO, unpublished data] in our organ culture studies. More extensive studies must be done to determine whether this enhancing effect is a unique specific property of estrogen or merely a general effect of hormones.

(b) Estrogen regulates carcinogen-RNA interaction. It appears logical to state a priori that when an effective dose of a chemical carcinogen is administered, the compound must necessarily reach the target cell in which an interaction between the compound and the cellular constituent occurs in order to initiate the neoplastic transformation. If so, what effects does estrogen exert in the interaction between the carcinogenic hydrocarbon and the cellular constituents of the mammary gland?

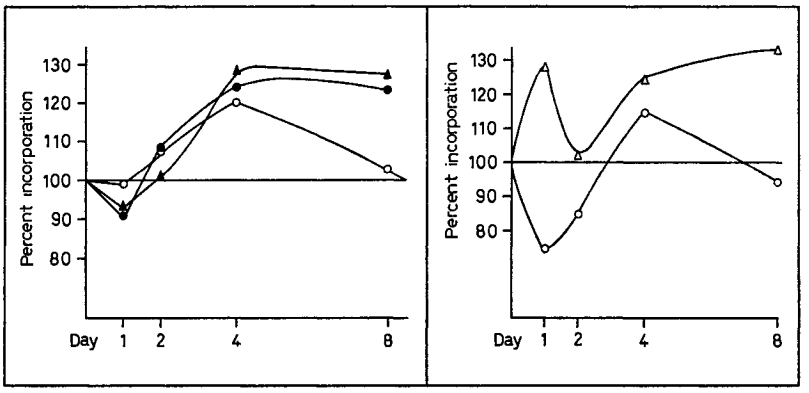
The early effects of a chemical carcinogen on the target cells cannot be elucidated by morphological studies, since submicroscopic changes are

not discernible at a very early stage. Recently, LIBBY and DAO [1966] studied the effects of DMBA on one-carbon metabolism in the mammary gland. We found that a single dose of 20 mg of DMBA induced a significant inhibition of the incorporation of ^{14}C-formate into the mammary gland RNA of female rats. The most interesting observation of this study was the finding that incorporation of the label into the RNA of the mammary gland was dependent on sex hormones. Whereas incorporation of ^{14}C-formate into mammary RNA was significantly inhibited in female rats receiving DMBA, it was markedly stimulated in male rats similarly treated. Administration of DMBA to castrated female rats failed to exert the inhibitory effect of incorporation of ^{14}C-formate into the RNA of the mammary gland. In castrated male rats, DMBA was unable to stimulate the incorporation of formate into mammary gland RNA.

When 10 μg of 17β-estradiol was injected daily for 7 days into the castrated female and male rats, a marked increase in formate incorporation was observed in both. After feeding of a single dose of DMBA to estradiol-treated castrated female and male rats, significant inhibition of ^{14}C-formate incorporation into mammary gland RNA was again observed in both. Using H^3-adenosine as a precursor, we repeated these experiments, with essentially similar results. In addition, when polycyclic aromatics, including phenanthrene, chrysene, and 3MC, were studied, we found that whereas 3MC and DMBA caused significant inhibition of RNA synthesis, phenanthrene and chrysene induced stimulation of RNA synthesis (fig. 1). When these compounds were studied in male rats, stimulation of mammary gland RNA synthesis was observed in all cases (fig. 2).

The inhibiting effect of DMBA on the incorporation of formate or adenosine is evidently a specific effect on the mammary gland. Incorporation of ^{14}C-formate into RNA is inhibited by DMBA in the mammary glands in female rats but is stimulated in the liver and kidneys in the same rats.

The effect of DMBA on DNA synthesis, however, was quite different. The incorporation of ^{3}H-thymidine into mammary gland DNA of female and male rats *in vitro* and *in vivo* and the DNA polymerase activity of mammary gland tissues were studied [TOMINAGA, LIBBY and DAO, 1970]. It was found that incorporation of ^{3}H-thymidine into the female mammary gland was severely inhibited at 6 h and at 1, 2, and 4 days after a single oral dose of 20 mg of DMBA. A similar inhibitory effect was observed in male rats, but the level of inhibition was less than in the female rats. Phenanthrene, a noncarcinogen for the mammary gland, exerted no effect



1, 2

Fig. 1. Effect of hydrocarbons on the incorporation of ^{3}H-adenosine into female rat mammary gland RNA (●–● 100 mg phenanthrene) (○–○ 20 mg benzpyrene) (▲–▲ 25 mg chrysene)

Fig. 2. Effect of 30 mg of 3-MC on the incorporation of ^{3}H-adenosine into RNA in the rat mammary gland. Incorporation is expressed as percent of control values. (○–○ female rats, △–△ male rats)

on ^{3}H-thymidine incorporation. Autoradiographic study of ^{3}H-thymidine incorporation into mammary gland DNA revealed a significant decrease in the labeling index at 6, 24, and 48 h after DMBA feeding. The grain-count distribution in the labeled cells showed that not only were fewer cells labeled after DMBA administration, but such cells also incorporated less thymidine per cell.

These data clearly show that the inhibitory effect of DMBA on thymidine incorporation into mammary gland DNA is virtually identical in male and female rats. Inasmuch as mammary cancer rarely develops in male rats but occurs with regularity in females after a single dose of DMBA, we conclude that the early inhibitory effect of DMBA on DNA synthesis in mammary gland cells is not related to the process of carcinogenesis. SHIMKIN, GRUENSTEIN, THATCHER and BASERGA [1967] also observed a striking decrease in DNA synthesis in epithelial cells of the breast and sebaceous glands within 24 h after a single feeding of 15 mg of DMBA in both male and female rats, yet carcinoma of the preauricular gland appeared ten times as frequently in males as in females [GRUENSTEIN, MERANZE and SHIMKIN, 1966; HUGGINS and GRAND, 1966].

IV. Metabolic Alteration of Carcinogenic Hydrocarbons and Inhibition of Tumor Induction

A. Effect of Hepatic Injury on Inhibition of Induction of Mammary Tumors by Chemical Carcinogenesis

Liver injury in rats can be induced by two methods: feeding of an unbalanced diet high in lipid and low in protein, to produce hepatic steatosis, or i.p. injection of CCl_4, to produce hepatic necrosis. TANAKA and DAO [1965] reported that rats fed a high-lipid, low-protein diet for 10 days showed a generalized weight loss involving the whole body and all endocrine and visceral organs. Although there was a marked reduction in ovarian weight (26%), no definite evidence of ovarian atrophy was observed histologically. The mammary glands, however, revealed atrophy of epithelial cells. Lost weight was rapidly recovered when the rats were returned to a regular diet.

The liver in rats on the hyperlipid diet showed profound fatty changes: the liver cells were filled with globular fat, the hepatic lobules were swollen, and the portal triad was compressed. When these rats were returned to the regular diet, globular fat disappeared from the liver cells within 5 days, and reversible changes seemed to be complete in 10 days.

Injection of carbon tetrachloride induced central lobular necrosis, nuclear degeneration of hepatic cells, and polymorphonuclear infiltration. Central necrosis occurred on the second day of CCl_4 injection, but regeneration followed later.

The incidences of mammary cancer in rats on the hyperlipid diet and fed 10 mg of DMBA or 30 mg of 3MC were significantly decreased [TANAKA and DAO, 1965]. CCl_4 injection in rats subsequently fed a single dose of DMBA, however, did not inhibit tumor induction, but the latent period of tumor development was markedly prolonged.

The effects of impairment of liver function by CCl_4 at the time of DMBA administration on the induction of mammary cancer have also been investigated by others [KERNOHAN, INGLIS and WHEATLEY, 1967]. These authors showed that the mammary tumor incidence in rats receiving 0.3 ml of a 50% solution of CCl_4 in olive oil i.p. 24 h prior to DMBA treatment was not lowered, but in fact was somewhat higher than in the control group (86% vs. 76%). In addition, they showed that the total number of tumors developing was considerably higher in the CCl_4-treated group than in the control group.

B. Inhibition of Mammary Tumor Induction in Rats Pretreated with Aromatic Hydrocarbons

Discovery of the protective activity of several aromatic hydrocarbons against induction of adrenal necrosis by DMBA [DAO and TANAKA, 1963] led to investigation whether pretreatment with these compounds in rats receiving DMBA could also inhibit the induction of mammary cancer in these rats. DAO [1964] investigated the effect of pretreatment with a single dose of phenanthrene (100 mg), benz*(a)*anthracene (10 mg), benzo*(a)*pyrene (10 mg), 3MC (10 mg), or DMBA (10 mg), on the induction of mammary cancer in rats subsequently fed a single dose of 20 mg of DMBA. All of these compounds are effective in protecting the adrenal cortex against the induction of necrosis by DMBA. The results showed that there was only a slight decrease in the incidence of mammary cancer in the rats pretreated with the protective compounds. The latent period of tumor appearance, however, was significantly prolonged.

HUGGINS, GRAND and FUKUNISHI [1964], however, reported that any of the 6 aromatic compounds, benz*(a)*anthracene (50 mg), 3,9-DMBA (5 mg), 6,8-DMBA (5 mg), chrysene (15 mg), retene (1-methyl-7-isopropylphenanthrene) (10 mg), and 6-aminochrysene (5 mg), if given daily for 10 to 16 doses to rats receiving at the same time 3 injections of DMBA i.v. 3 days apart from each injection of 1 of the 6 compounds, prevented mammary cancer induction in a significant number of rats. In addition, the latent period of tumor appearance was significantly prolonged and the active centers were markedly reduced in the rats developing mammary cancers. These authors also showed that if the protective compounds were given in only a single dose and were followed by 3 injections of DMBA, mammary cancer induction was not inhibited.

WHEATLEY [1968] reported inhibition of mammary tumor induction by pretreatment with 1 mg of 3MC i.p. 24 h prior to injection of 2.5 mg of DMBA i.v. This effect was completely nullified, however, if the dose of DMBA was increased to 5.0 mg i.v. MILLER, MACDONALD and MILLER [1954] reported that the addition of 3MC to a diet containing 2-acetylaminofluorene or its highly carcinogenic 7-fluoro derivative resulted in a considerable reduction in the number of tumors of the mammary gland, liver, intestine, and ear duct.

C. Inhibition of Induction of Mammary Tumors by Phenothiazines and β-Naphthoflavine

WATTENBERG and LEONG [1967, 1968] reported inhibition of DMBA-induced mammary tumorigenesis by phenothiazine and β-naphthoflavine. These authors showed that administration of phenothiazine 48 h prior to oral administration of 2 to 4 mg of DMBA for 3 doses at 6-hour intervals inhibited mammary tumor induction (ca. 50%). If DMBA was given at a maximal dose of 20 mg, the inhibitory effect was completely abolished. Oral feeding of β-naphthoflavine (0.1 mmol in 1 ml of DMSO solution) 48 h prior to a single oral feeding of 12 mg of DMBA induced a 50% inhibition of mammary tumor induction in rats.

D. Discussion

Most, if not all, chemical carcinogens are probably deactivated metabolically to a greater or lesser extent. Polycyclic aromatic hydrocarbons, when administered to animals, are rapidly transformed into inactive forms and excreted. There has been no evidence to show that potent mammary carcinogens such as DMBA and 3MC must be activated metabolically for their actions. The unchanged DMBA and 3MC are likely the ultimate carcinogens for mammary carcinogenesis.

The foregoing experiments, with the exception of one by TANAKA and DAO [1965], all seem to suggest that the inhibition of mammary tumor induction by pretreating the rats with agents capable of inducing hepatic microsomal drug-metabolizing enzymes is due to metabolic inactivation of the carcinogen. WHEATLEY [1968] reported that pretreatment with 3MC together with an active inhibitor of drug metabolism (SKF 525A, β-diethylaminoethyldiphenyl-*n*-propyl acetate) results in enhancement of induction of mammary cancer in DMBA-treated rats. SKF 525A is known to be a potent inhibitor of hepatic microsomal enzyme systems [COOPER, AXELROD and BRODIE, 1954]. It has also been demonstrated that SKF 525A inhibits the metabolic transformation of DMBA to 7-hydroxymethyl-12-methylbenz*(a)*anthracene, which is an active adenocorticolytic compound [WHEATLEY, 1968]. Even so, neither polycyclic aromatic nor phenothiazine compounds can effectively inhibit the induction of mammary tumor if an optimal dose of DMBA is used as the carcinogen. The experiments of HUGGINS and FUKUNISHI [1964] demonstrated more effective in-

hibition of tumor induction if repeated injections of the protective agents were given to rats treated with DMBA as a carcinogen. This method, however, still failed to produce total inhibition. These data strongly suggest that there may be a difference of metabolism between the liver and the target tissue, which is, in this case, the mammary gland.

Earlier work by HUGGINS and FUKUNISHI [1964], and by DAO and VARELA [1966] both suggest that large amounts of DMBA are not rapidly inactivated by microsomal hydroxylating enzymes as a result of pretreatment with 3MC or other polycyclic aromatic hydrocarbons. DAO and VARELA [1966] showed that sequential administration of 3MC+DMBA and DMBA+DMBA induced the synthesis of amounts of BP hydroxylase larger than the sums of the amounts synthesized after corresponding single doses. HUGGINS and FUKUNISHI [1964] reported a similar observation on measuring menadione reductase in the liver after treatment with 3MC and DMBA. These authors found that rats that received 3MC+DMBA had a far greater increase in hepatic menadione reductase (376%) than did rats that received 3MC alone (276%).

Numerous experiments have been reported to suggest that in mammary carcinogenesis induced by DMBA and other polycyclic aromatic hydrocarbons, the carcinogenic hydrocarbon itself is responsible for mammary tumor induction. The evidence from transplantation experiments of DAO, TANAKA and GAWLAK [1964] and of DAO, KING and GAWLAK [1968] strongly suggest 'direct action' of the carcinogen on the target tissue. In these experiments, mammary cancer could develop in transplants as soon as 10 min after exposure to the carcinogen in the donor host. These observations almost conclusively rule out the possibility that DMBA must be metabolically activated in the liver before it exerts its action. The experiments of WHEATLEY [1968] using SKF 525A to enhance the development of mammary cancer in DMBA-treated rats provide further evidence to support the postulate that DMBA, itself not a metabolite, is the ultimate carcinogen. Perhaps the most convincing experiment is the *in vitro* exposure of the mammary gland to DMBA and the subsequent development of mammary cancer in it [DAO, 1970]. In this experiment, rat mammary glands that were excised and incubated for 10 min in a medium containing DMBA developed mammary adenocarcinoma after reimplantation in the intrascapular region of the back. The mammary tumor incidence in the autologously transplanted grafts was parallel to the concentration of the carcinogen in the graft. The results indicate that the carcinogenic effect of DMBA in the mammary gland is 'direct' and not via the host.

Even so, it would be naive to conclude that the 'unchanged DMBA' is necessarily the ultimate carcinogen. The findings of MILLER, MILLER and HARTMANN [1961] have clearly demonstrated the importance of the proximate carcinogenic metabolites. Polycyclic aromatic hydrocarbons are inert, unreactive compounds. Metabolic activation may be required to convert a carcinogen into an ultimate reactive and carcinogen form. The recent work of DEBAUM, SMITH, MILLER and MILLER [1970] demonstrating the sulfation of N-hydroxy-AAF *in vivo* is indicative of this mechanism.

If DMBA must necessarily be metabolically activated for its action, then this activation must occur in the mammary gland. It is imperative that the inactivation or activation of a chemical carcinogen be studied in the target tissue. The mammary gland is sensitive to the action of carcinogenic aromatic polycyclic hydrocarbons, and is a major site of concentration of the carcinogenic hydrocarbons, which must necessarily be metabolized either for deactivation or activation. Such a study has not been made. Preliminary results of investigation in my laboratory show that the patterns of metabolism of DMBA in the liver and the mammary gland are quite different [DAO and TAMULSKI, unpublished data). We found that the liver metabolized DMBA to noncarcinogenic 7,12-dihydroxymethylbenz*(a)*anthracene much more efficiently than did the mammary gland, whereas the mammary gland was much more active in metabolizing DMBA to 12-hydroxymethyl-7-methylbenz*(a)*anthracene which is also noncarcinogenic. The rate of transformation to these noncarcinogenic metabolites is significantly greater in the liver than in the mammary gland. Both the liver and the mammary gland form 7-hydroxymethyl-12-methylbenz*(a)*anthracene, but the magnitude of transformation is very small.

Liver damage as a result of CCl_4 injection failed to alter the incidence of mammary cancer in rats treated with DMBA. It is apparent that the damage inflicted upon the liver by CCl_4 was not sufficiently great to depress the synthesis of metabolizing enzymes for metabolic inactivation of DMBA; hence, there was an increase in tumor incidence. In contrast, fatty degeneration of liver parenchymal cells caused by feeding a hyperlipid diet caused dysfunction of endocrine glands and atrophy of the mammary gland, and consequently a significant decrease in mammary tumor induction.

V. Recapitulation and Conclusions

Inhibition of chemical carcinogenesis in experimental animals has been studied in several tissues including skin, liver and mammary gland.

In this chapter, experiments are presented in which inhibition of tumor induction in the mammary gland by chemical carcinogen has been achieved by the following methods: (a) hormonal stimulation of target tissue; (b) hormonal deprivation of the host; and (c) administration of 1 of a variety of chemical compounds capable of stimulating drug-metabolizing enzyme systems.

A. Induction of Metabolizing Enzymes

The inhibitory chemical compounds discussed in this paper bring about the inhibitory effect by stimulating the capacity of the animals to inactivate or detoxify the chemical carcinogens, which normally are not present in the organism. The drug-metabolizing enzyme systems have been studied extensively.

These microsomal metabolizing enzyme systems, requiring NADPH and O_2 for activity, can detoxify not only drugs but also a variety of chemical carcinogens, including polycyclic aromatic hydrocarbons [CONNEY, 1957; COOK and SCHOENTAL, 1952], azo dyes [CONNEY, MILLER and MILLER, 1956], aromatic amines [CRAMER, MILLER and MILLER, 1960]. All these studies deal mainly with the induction of hepatic microsomal enzyme systems. Recently, studies show that tissues other than liver also possess detoxification enzyme systems. Tissues such as the gastrointestinal tract [WATTENBERG, LEONG and STRAND, 1962], lung and kidney [GELBOIN and BLACKBURN, 1964; WATTENBERG and LEONG, 1965] are found to possess the enzyme systems which can be induced by chemical carcinogens. DAO and VARELA [1966] examined several tissues for microsomal metabolizing enzymes but found that the mammary and adrenal glands have very low levels of benzpyrene hydroxylase activity; furthermore, pretreatment with 3MC failed to stimulate the synthesis of the enzyme. This fact is interesting since the mammary and adrenal glands of rats are most susceptible to DMBA injury. Cancer invariably develops in the mammary gland and necrosis and hemorrhage are regularly observed in the adrenal gland following DMBA treatment.

The inhibitory effect of many polycyclic hydrocarbons in inhibiting the effect of DMBA on induction of adrenal necrosis and mammary tumor is no doubt due, in part, to a metabolic detoxification of DMBA by the hepatic microsomal metabolizing enzymes. Induction of metabolizing enzyme synthesis and inhibition of tumorigenesis in many instances is rather nonspecific. For instance, a very small dose of a potent inducer such as 3MC can induce a re-

markable increase of hepatic microsomal metabolizing enzyme for hydroxylating compounds such as polycyclic aromatic hydrocarbons, aromatic amines and azo dyes and can inhibit tumor induction in liver by 3-methyl-DAB [MEECHAN, MCCAFFERTY and JONES, 1953], epidermoid carcinogenesis by aromatic amines [MILLER, MILLER, BROWN and MACDONALD, 1958]. The inhibitory effect of 3MC on mammary tumor induction by DMBA is not entirely clear. It does not appear that the mechanism is merely an induction of microsomal enzymes by the inhibitors [DAO and VARELA, 1966; HUGGINS and FUKUNISHI, 1964]. The key to answer the question is to study the DMBA hydroxylating enzymes in the target tissue. Since in this case the mammary gland is the target tissue, metabolism of DMBA in the mammary gland must be investigated.

B. Competitive Inhibitory Mechanism

The efficacy of polycyclics to inhibit the induction of adrenal necrosis and mammary tumor by DMBA appears to bear some relationship to the molecular similarity of the inhibitors and the carcinogen. DAO [1964] demonstrated that compounds closely related to DMBA structurally such as 3MC, DBA, BP and BA were significantly more active in protecting the adrenal cortex against injury induced by DMBA than those which are less structurally related such as fluorene, acridine, and naphthalene. Similar observation has been made in the effect of certain polycyclic aromatics in inhibiting DMBA-induced epidermal carcinogenesis [HILL, STANGER, PIZZO, RIEGEL, SHUBIK and WARTMAN, 1951].

Although the structural similarity between the inhibitor and the carcinogen often suggests the possibility of competitive inhibition, conclusive evidence has not been presented. In studies on the inhibition of mammary tumor induction by DMBA, it was found that 3MC given 24 h prior to DMBA administration inhibits mammary tumor induction by 2.5 mg DMBA, but has no effect if given 1 h before DMBA. This observation suggests that 3MC is not competing with DMBA for binding sites in the mammary epithelial cells to achieve protection.

In contrast, our experiments show that ovarian hormones available in large amounts either by introduction of pregnancy or injections of the hormones shortly before carcinogen administration can effectively inhibit tumor induction. The unique structural similarity between steroid hormone and polycyclic aromatic hydrocarbon led to the suggestion that ster-

oid hormone and carcinogenic polycyclics may compete for tissue-binding sites.

Experiments presented in this paper almost uniformly show that to achieve effective inhibition of mammary tumor induction by DMBA, large amounts of steroid hormone must be either injected or made available by pregnancy before DMBA administration. This fact seems to support the postulate that one mechanism in the inhibition of tumor induction is saturation of tissue-binding sites by steroid hormone to prevent subsequent binding with DMBA. Sufficient proof of this mechanism of inhibition however is not available.

C. Interference with Interaction between Carcinogen and Target Cells

The experiments concerned with inhibition of tumor induction in castrated female rats provide conclusive evidence that hormones from the ovary, specifically the estrogens, are of critical importance in the initiation phase of carcinogenesis in the mammary gland. The results give convincing evidence that hormone may control the regulatory mechanism for interaction between carcinogen and the target tissue.

Our biochemical studies reveal that one of the early events after treatment with a carcinogen is the alteration of RNA metabolism in the mammary gland by the carcinogen. Perhaps the observations of great significance are: (a) the inhibition of RNA synthesis in the mammary gland of DMBA-treated rats is estrogen-dependent; and (b) noncarcinogens, even with similar chemical structures, fail to cause any effect on RNA metabolism in the mammary gland.

Our experiments disclose that administration of small amounts of estrogen (10 μg daily for 7 days) to castrated female rats restores the inhibitory effect of DMBA on RNA synthesis which is lost when ovaries are removed. From our histological studies, small doses of estrogen injected for a short period of time do not induce any observable morphological or functional changes in the mammary epithelium. One interesting phenomenon is the observation that the markedly reduced mitogenic activity of the mammary epithelial cells caused by castration has been augmented after estrogenic treatment. It can be concluded that if interaction between carcinogen and mammary macromolecules must necessarily be the initial requisite for carcinogenesis in the mammary gland, such an interaction must occur when cells are in mitosis. Estrogen perhaps acts to maintain the mi-

togenic activity of the mammary cells. Cells not in mitosis or those in rapid mitosis (hyperplasia) are protected from injury by a carcinogen.

Our experiments with mammary gland transplantations [DAO, TANAKA and GAWLAK, 1964] show that in allogenic transplantation, the effect of carcinogen (DMBA) on the transplantability of the skin and mammary gland is markedly different. The study shows that a single dose of 3MC or DMBA given prior to cross skin grafting in random-bred Sprague-Dawley rats fails to prolong or increase survival of the skin grafts. In contrast, pretreatment of the donor rats with DMBA significantly favored survival of the grafts in the recipient allogenic hosts. Whereas only 20% of the mammary glands from the controls survived after transplantation to the recipients, between 78 and 93% were surviving at the end of 6 months, if the grafts were made 10 days after a single dose of DMBA to donor rats before transplantation. These results again suggest that skin, having very slow mitotic activity, is much less susceptible to carcinogen attack than the mammary gland which has more active mitogenic activity. It was further demonstrated by our experiments that the survival of mammary gland graft from castrated female rats receiving DMBA treatment is only comparable to that of the skin graft [DAO, 1966]. It seems clear that whatever the effect of DMBA on the transplantability of the mammary gland may be, it is lost in the absence of estrogen.

Evidence that alteration of DNA-dependent RNA synthesis may be involved in the initiation phase of carcinogenesis is also provided by the experiments of inhibition of epidermal carcinogenesis by actinomycin D [GELBOIN and KLEIN, 1964]. These authors show that actinomycin D, a DNA-dependent RNA synthesis inhibitor, when given to mice prior to a single application of DMBA to the skin, significantly inhibited the induction of skin tumors. In our laboratory similar investigation has been carried out to study the effect in carcinogenesis in the mammary gland.

D. Significance

Studies of inhibition of carcinogenesis may lead to a better understanding of the hitherto poorly understood mechanism of carcinogenesis. Many postulated mechanisms of carcinogenesis are often difficult to prove with direct experiments, but indirect experiments can be designed to elucidate certain concepts. For instance, the study of inhibition of epidermal carcinogenesis by actinomycin D suggests that the initiation of carcinogenesis may in-

deed involve an alteration of DNA-dependent RNA synthesis which can be blocked. Estrogen has been generally considered as a promoting agent, but the tumor inhibition experiments presented in this chapter strongly suggest that it is critically needed in the initiation phase, suggesting its dual function. These studies represent some advance in the knowledge of carcinogenesis.

The significance of these studies in relation to human breast cancer cannot be clearly stated. First, there is no epidemiological evidence to indicate that chemical carcinogens are indeed involved in the genesis of breast cancer in women. Second, extrapolation of data from the experimental system to the human situation is difficult and not clear-cut. The implications, however, are clear that whatever the carcinogenic agents are, be they chemical agents, viruses, X-ray or others, the ultimate changes from normal mammary cells to the neoplastic form must require the presence of estrogens—an endogenous hormone that regulates the activity of the target tissue during the life-span of the host. Carcinogenesis is a slow process; the initiation phase occurs early in man's life and progresses to the visible tumor at various times, from during the reproductive age to the period of aging. This fact has been demonstrated by our experiments dealing with carcinogenesis in relation to age [DAO, 1969]. Our experiments have also unequivocally shown that the amount of carcinogenic agent needed to induce neoplastic changes in the mammary gland is infinitely small and the induction period is relatively long. The process of carcinogenesis is clearly reversible. The question is, at what stage?

Of all the methods for inhibition of tumor induction described in this chapter, the most effective is the induction of mammary hyperplasia prior to carcinogen administration. It seems that the functionally active mammary gland is refractory to carcinogenesis. The epidemiological studies of human breast cancer have, for a long time, suggested that the incidence of breast cancer is lower in parous women and in women who have a history of long-term nursing [LEVINE, SHEEHE, GRAHAM and GLIDEWELL, 1964]. The implications are that women with repeated pregnancies and lactation are more refractory to the development of breast cancer. These observations in humans are in agreement with experimental data reported in this chapter. The evidence provides a sound basis for studies in humans. Conceivably, methods for the inhibition of tumorigenesis in humans can be derived from these experimental studies. It seems quite possible that the incidence of mammary cancer can be greatly reduced, even if total prevention is not achieved.

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