1971

Promotion Of 7,12-dimethylbenz(α)anthracene-induced Mammary Cancer In Female Sprague-Dawley Rats By Dietary Fats

Hun-teik Khor

Follow this and additional works at: https://ir.lib.uwo.ca/digitizedtheses

Recommended Citation
Khor, Hun-teik, "Promotion Of 7,12-dimethylbenz(α)anthracene-induced Mammary Cancer In Female Sprague-Dawley Rats By Dietary Fats" (1971). Digitized Theses. 548.
https://ir.lib.uwo.ca/digitizedtheses/548

This Dissertation is brought to you for free and open access by the Digitized Special Collections at Scholarship@Western. It has been accepted for inclusion in Digitized Theses by an authorized administrator of Scholarship@Western. For more information, please contact tadam@uwo.ca, wlswadmin@uwo.ca.
Promotion of 7,12-Dimethylbenz(a)anthracene-Induced Mammary Cancer in Female Sprague-Dawley Rats by Dietary Fats

by

Hun-Teik Khor

Department of Biochemistry

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario, Canada.

August, 1971.

© Hun-Teik Khor 1971
This study was supported by the National Cancer Institute of Canada. The author wishes to express his gratitude to the organization.
ACKNOWLEDGEMENTS

The author wishes to take this opportunity to express his sincere gratitude to Professor K. K. Carroll for his patient guidance, valuable discussions and comments during the course of this study.

The author also wants to thank Proctor and Gamble Ltd., Hamilton, Ontario for supplying a number of fats and oils used in this study and Dr. Paul E. Schurr for the gift of DMBA-Fat emulsions.

Gratefulness is extended to Mr. R. Rasmussen for the general care of the animals, to Mr. H. E. Pedersen for certain technical assistance and to Mrs. A. Varga for preparing the tumor slides. The author would like to express his appreciation to his colleagues for their cooperation in many ways during the past few years.
CONTENTS

Acknowledgements .................................................. iv
List of Tables ...................................................... viii
List of Figures .................................................... x
Abstract .................................................................. xii

INTRODUCTION ................................................................ 1

REVIEW OF LITERATURE

I. EXPERIMENTAL INDUCTION OF MAMMARY CANCER IN LABORATORY
   ANIMALS ................................................................ 5
II. THE TWO-STAGE MECHANISM OF CARCINOGENESIS ............... 8
III. ACCUMULATION AND CLEARANCE OF POLYCYCLIC HYDROCARBONS
    IN MAMMARY TISSUE ............................................ 10
IV. METABOLISM OF DMBA AND THE PROXIMATE CARCINOGEN ...... 14
V. DMBA-INDUCED ADRENAL NECROSIS AND ITS IMPLICATIONS
   ON MAMMARY CARCINOGENESIS ............................... 16
VI. FACTORS AFFECTING MAMMARY CARCINOGENESIS IN THE RAT .................. 24
VII. HORMONAL FACTORS IN MAMMARY CARCINOGENESIS IN RATS ............. 25
    1. Involvement of Hormones in the Initiation of
       Mammary Tumors .............................................. 25
    2. Involvement of Hormones in the Development, Growth
       and Maintenance of Mammary Tumors ................. 31
VIII. EFFECTS OF DIETARY FAT ON CHEMICAL CARCINOGENESIS IN
      MICE AND RATS .............................................. 38
      1. Influences of Dietary Fats on Various Types of
         Tumors ...................................................... 39
2. Mode of Action of Dietary Fat on Tumor Formation —— 42

IX. DISTRIBUTION OF LABELLED ESTRADIOL IN VARIOUS TISSUES
AFTER INJECTION ------------------------------------------ 45

MATERIALS AND METHODS

I. MATERIALS --------------------------------------------- 49
A. Animals ------------------------------------------------ 49
B. Chemicals, Solvents and Reagents ------------------------ 49

II. METHODS ----------------------------------------------- 50
A. Long-term feeding experiments -------------------------- 50
B. Biochemical Analysis ----------------------------------- 51
1. Determination of the Apparent Digestibility of Dietary Fat ------------------------------------------ 51
2. Determination of Fatty Acid Composition of Fats and Oils -------------------------------------------- 52
3. The Distribution of Radioactivity in Rat Tissues after Injection of Tritiated Steroid Hormones ------- 53
4. Metabolism of Estradiol-4-14C in the Liver in vivo ---- 54
5. Analysis of Plasma Lipids ------------------------------- 55

III. STATISTICAL METHODS ---------------------------------- 58

EXPERIMENTAL RESULTS
Effect of Different Levels of Dietary Fat on Mammary Tumor Incidence ----------------------------------- 59
Effect of Different Types of Dietary Fat on Mammary Tumor Incidence ----------------------------------- 65
Effect of Dietary Fat on Mammary Carcinogenesis induced by Intravenous Injection of DMBA ---------------- 71
Mammary Carcinogenesis in Female Rats Switched to High Corn Oil Diet at Different Intervals after DMBA Administration ------------------------------------------ 78
Mammary Carcinogenesis in Female Rats switched to Low Corn Oil Diet at Different Intervals after DMBA Administration ........................................ 85

Mammary Carcinogenesis in Female Rats switched to Low Corn Oil Diet Two Months after DMBA Administration ........... 90

The Distribution of Radioactivity in Tissues of 50-60 day old Female Rats after Injection of Tritiated Estradiol-17β ....................................... 93

The Distribution of Radioactivity in Tissues of 90-100 day old Female Rats after Intravenous Injection of Tritiated Estradiol-17β ........................................ 96

The Distribution of Radioactivity in Tissues of 90-100 day old Female Rats after Intravenous Injection of Tritiated Progesterone ........................................ 98

Metabolism of Estradiol-4-14C in the Liver of Female Rats in Vivo ...................................................................................................................... 100

Effect of Dietary Fat on the Level of Plasma Lipids in Female Rats .............................................................................................................. 102

DISCUSSION .................................................................................................................. 105

SUMMARY AND CONCLUSIONS ............................................................................ 125

REFERENCES ........................................................................................................... 129

APPENDIX I ............................................................................................................. 164

VITA .......................................................................................................................... 165
LIST OF TABLES

Table:                                                                                   Page

I. Mammary tumor incidence following administration of DMBA to rats on semisynthetic diets containing different levels of corn oil .............................. 62

II. Incidence of different types of mammary tumors in rats on semisynthetic diets containing different levels of corn oil. ...................................................... 63

III. Mammary tumor incidence following administration of DMBA to rats on diets containing different fats and oils at the level of 20% by weight of the diet. .............. 68

IV. Incidence of different types of mammary tumors following administration of DMBA to rats on diets containing different fats and oils. ...................................... 69

V. Fatty acid composition of fats and oils. ................................................................. 70

VI. Effect of dietary fat on mammary tumor incidence induced in female rats by a single dose of 5 mg. of DMBA injected intravenously. ........................................... 75

VII. Effect of dietary fat on the incidence of different types of mammary tumors induced in female rats by a single dose of 5 mg. of DMBA injected intravenously. ............. 76

VIII. Effect of changing from low corn oil (5% by weight) diet to high corn oil (20% by weight) diet at different intervals after DMBA administration on mammary tumor incidence in rats. .............................................. 81

IX. Effect of changing from low corn oil (5% by weight) diet to high corn oil (20% by weight) diet at different intervals after DMBA administration on the incidence of different types of mammary tumors in rats. ..................................... 82
X. Effect of switching from high corn oil (20% by weight) diet to low corn oil (5% by weight) diet at different intervals after DMBA administration on mammary tumor incidence in rats. 88

XI. Effect of switching from high corn oil (20% by weight) diet to low corn oil (5% by weight) diet at different intervals after DMBA administration on the incidence of different types of mammary tumors in rats. 89

XII. Effect of switching rats from high corn oil (20% by weight) diet to low corn oil (5% by weight) diet two months after DMBA administration on mammary tumor incidence. 92

XIII. Metabolism of estradiol-4-$^{14}$C in the liver of female rats fed on high and low corn oil diets. 101

XIV. Effect of dietary corn oil on the level of plasma lipids in female rats. 103
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Effect of diets containing different levels of corn oil on the cumulative palpable mammary tumor incidence in female rats treated with DMBA</td>
<td>60</td>
</tr>
<tr>
<td>2.</td>
<td>Effect of diets containing different levels of corn oil on the rate of appearance of palpable mammary tumors in female rats treated with DMBA</td>
<td>64</td>
</tr>
<tr>
<td>3.</td>
<td>Effect of different dietary fats and oils fed at the level of 20% by weight of the diet on the cumulative palpable mammary tumor incidence in female rats treated with DMBA</td>
<td>66</td>
</tr>
<tr>
<td>4.</td>
<td>Effect of different dietary fats and oils fed at the level of 20% by weight of the diet on the rate of appearance of palpable mammary tumors in female rats treated with DMBA</td>
<td>72</td>
</tr>
<tr>
<td>5.</td>
<td>Effect of high fat diet (20% corn oil) and low fat diet (5% corn oil) on the cumulative palpable mammary tumor incidence induced in female rats by a single intravenous injection of 5 mg. of DMBA-fat emulsion</td>
<td>74</td>
</tr>
<tr>
<td>6.</td>
<td>Effect of high fat diet (20% corn oil) and low fat diet (5% corn oil) on the rate of appearance of palpable mammary tumors in female rats induced by a single intravenous injection of 5 mg. of DMBA-fat emulsion</td>
<td>77</td>
</tr>
<tr>
<td>7.</td>
<td>Effect of switching female rats from low fat diet (5% corn oil) to high fat diet (20% corn oil) at different intervals after DMBA administration on the cumulative palpable mammary tumor incidence</td>
<td>79</td>
</tr>
<tr>
<td>8.</td>
<td>Effect of switching female rats from low fat diet (5% corn oil) to high fat diet (20% corn oil) at different intervals after DMBA administration on the cumulative palpable mammary tumor incidence</td>
<td></td>
</tr>
</tbody>
</table>
corn oil) to high fat diet (20% corn oil) at different intervals after DMBA administration on the rate of appearance of palpable mammary tumors 84

9. Effect of switching female rats from high fat diet (20% corn oil) to low fat diet (5% corn oil) at different intervals after DMBA administration on the cumulative palpable mammary tumor incidence 86

10. Effect of switching female rats from high fat diet (20% corn oil) to low fat diet (5% corn oil) at different intervals after DMBA administration on the rate of appearance of palpable mammary tumors 91

11. Effect of high fat diet (20% corn oil) and low fat diet (5% corn oil) on the distribution of radioactivity in various tissues of female rats after intramuscular injection of tritiated estradiol-17β. (50-60 day old rats) 94

12. Effect of high fat diet (20% corn oil) and low fat diet (5% corn oil) on the distribution of radioactivity in various tissues of female rats after intravenous injection of tritiated estradiol-17β. (50-60 day old rats) 95

13. Effect of high fat diet (20% corn oil) and low fat diet (5% corn oil) on the distribution of radioactivity in various tissues of female rats after intravenous injection of tritiated estradiol-17β. (90-100 day old rats) 97

14. Effect of high fat diet (20% corn oil) and low fat diet (5% corn oil) on the distribution of radioactivity in various tissues of female rats after intravenous injection of tritiated progesterone. (90-100 day old rats) 99
ABSTRACT

Results obtained in the present studies confirmed previous findings that a semisynthetic diet containing 20% corn oil enhanced the yield of mammary tumors induced in female Sprague-Dawley rats by 7,12-dimethylbenz(a)anthracene (DMBA) compared to a similar diet containing 0.5% corn oil and provided additional information on the effects of intermediate levels of dietary fat on mammary carcinogenesis. Increasing the level of corn oil from 0.5% to 5% did not increase the tumor yield, but raising the level from 5% to 10% increased it significantly. Increasing the level of corn oil from 10% to 20% did not result in further increase in tumor yield.

The tumor-promoting property of different saturated and unsaturated fats and oils in rats was compared by feeding them at the level of 20% by weight of the diet. Although the percentage of rats bearing mammary tumors was not much different among the different groups, there was an apparent trend for those on diets containing unsaturated fats to have more mammary tumors than those on diets containing saturated fats. However, rapeseed oil was an exception to this generalization.

Experiments carried out to study the mechanism of action of dietary fat indicated that its promoting effect on mammary carcinogenesis could be observed whether the carcinogen was given intragastrically or intravenously. Previous findings showed that high corn oil diet provoked as many mammary tumors when started one day after carcinogen treatment as when started four weeks before treatment. Further experi-
ments showed that high corn oil diet started one week after carcinogen treatment produced as many mammary tumors as before. However, when started two weeks after, its promoting effect was somewhat diminished and at four weeks after carcinogen treatment, it produced no effect on the tumor yield.

Conversely, low corn oil diet appeared to be more effective in lowering the tumor yield when fed to rats beginning one week after carcinogen treatment than when it was started two or four weeks after treatment. Low corn oil diet fed to rats beginning two months after carcinogen treatment did not appear to affect the growth of existing mammary tumors. Results obtained in the preceding experiments support our previous conclusion that high fat diet affects the development rather than initiation of mammary tumors. The significance of these findings was discussed in relation to human breast cancer.

Studies on the distribution of radioactivity in female rats after injection of tritiated estradiol indicated that rats on low corn oil diet tended to accumulate more radioactivity in their tissues than those on high corn oil diet. This difference appeared to be due to differences in metabolism of estradiol in the liver. When tritiated progesterone was used, no difference in uptake of radioactivity could be seen.

Analysis of plasma lipids indicated that rats on high corn oil diet had significantly lower plasma triglyceride level and higher plasma free fatty acid level than those on low corn oil diet.
INTRODUCTION

Experiments carried out in a number of different laboratories over the past 30 years have provided evidence that rats and mice on high fat diets are more prone to develop mammary tumors than control animals on low fat diet (Tannenbaum, 1959; Carroll, Gammal and Plunkett, 1968). This applies to both spontaneous tumors and tumors induced by various means (Dunning, Curtis and Maun, 1949; Engel and Copeland, 1951; Gammal, Carroll and Plunkett, 1967; Carroll and Khor, 1970), and the effects seem not to be dependent on differences in caloric intake (Silverstone and Tannenbaum, 1950; Gammal et al, 1967).

Examination of statistical data for humans reveals a strong positive correlation between fat intake and mortality from breast cancer in different countries of the world (Lean and Birm, 1966; Carroll et al, 1968). The possible involvement of dietary fat in the aetiology and development of human breast cancer has received increasing attention in recent years (Wynder, 1969; Hems, 1970). The aim of the studies to be reported in this thesis was to find out how dietary fat affects mammary carcinogenesis in the rat with the hope that the results obtained with laboratory animals might shed light on the aetiology and development of human breast cancer.

Previous studies in our laboratory showed that rats on high fat semisynthetic diet containing 20% corn oil developed more mammary tumors than rats on corresponding diets containing either 20% coconut oil or 0.5% corn oil (Gammal et al, 1967). In subsequent studies, the level of
carcinogen in mammary tissue was measured at different time intervals after carcinogen administration, and although the average level was somewhat higher on the 20% corn oil diet during the first 12 hours, it seems doubtful that the difference was sufficient to account for the higher tumor yield (Gammal, Carroll and Plunkett, 1968). However, there have been no systematic studies in the literature on the effects of different fats and oils on mammary carcinogenesis and the objective of the first long-term study was to determine the effects of intermediate levels of corn oil on mammary carcinogenesis and to assess the effects of other dietary fats and oils under the same experimental conditions.

All our previous studies (Gammal et al, 1967; Carroll and Khor, 1970) have been carried out with mammary tumors induced by a single administration of 7,12-dimethylbenz(a)anthracene (DMBA) by stomach tube. It is known that the route of administration of the carcinogen could affect the outcome of the experiment (Huggins and Fukumishi, 1963b). An experiment was therefore carried out to investigate whether or not the promoting effect of dietary fat on mammary carcinogenesis could be demonstrated when the carcinogen was given intravenously.

Carroll and Khor (1970) have shown that female rats which received high corn oil diet beginning one day after DMBA administration produced as many mammary tumors as those fed on high corn oil diet throughout the experimental period. Likewise, low corn oil diet when fed to animals beginning one day after carcinogen treatment lowered the yield of mammary tumors to the same extent as the same diet fed to the animals immediately after weaning. These results suggested that the
effects of dietary fat was related mainly to the developmental stage rather than the initiation stage of the carcinogenic process. In order to gain further insight into this problem, experiments were carried out to study the effects of transferring female rats from high corn oil diet to low corn oil diet at different intervals after DMBA treatment on mammary carcinogenesis and vice versa. These experiments will provide further evidence for our earlier conclusion that high fat diet affected mainly the development of mammary tumors.

Although the promoting effect of dietary fat on mammary carcinogenesis in rats and mice has been observed since the early 1940's, the mechanism of action of dietary fat remains largely unknown. Dunning et al (1949) and Benson, LeV and Grand (1956) noted in histological sections that rat mammary gland appeared more active in animals on high fat diet compared to those from rats on low fat diet. The cause of the difference in structure of the mammary gland of rats fed on diets containing high and low fat content is not known. However, in view of the fact that estrogen is deeply involved in the development of mammary tumors (Dao, 1964a, 1969a) and that estrogen can modify the metabolic functions of tissues and cells (Grant, 1969), it seemed possible that high fat diet might alter the distribution and metabolism of estrogen and thus alter the hormonal level in the mammary tissue. To test this hypothesis, tritiated estradiol-17β was injected into female rats which has been fed on high or low corn oil diet for several weeks and the distribution of the labelled estradiol was determined.

After the completion of the above experiment, it was found
that rats on low corn oil diet accumulated more radioactivity in the pituitary, ovaries, adrenals, uterus and mammary gland than those fed on high corn oil diet. Since it has been repeatedly confirmed by a number of investigators (Jensen and Jacobson, 1962; King, Gordon and Inman, 1965b; Sander, 1968; Puca and Bresciani, 1968) that over 90% of the radioactivity accumulated in the pituitary, uterus and mammary gland is accounted for by the intact labelled estradiol, it seemed possible that the difference in the uptake of radioactivity between the two dietary groups might be due to differences in the metabolism of this steroid in the liver. A preliminary experiment was therefore performed to check this possibility.

The involvement of progesterone in the promotion of mammary carcinogenesis is well established (Dao, 1964a, 1969a). Whether progesterone acts directly on the mammary gland or indirectly through the pituitary to result in promotion of mammary carcinogenesis is not known. Since it was found that the distribution and metabolism of labelled estradiol were quite different in rats fed on high and low fat (corn oil) diets, it was considered of interest to see how tritiated progesterone was distributed in different tissues after an intravenous injection. An experiment was carried out with this objective in mind.

Analysis of plasma lipids of female rats fed on high and low fat diets also carried out to see if the level of dietary fat in the diet might have any effect on the composition of plasma lipids.
REVIEW OF LITERATURE

I. EXPERIMENTAL INDUCTION OF MAMMARY CANCER IN LABORATORY ANIMALS.

The story of chemical carcinogenesis probably began with Percivall Pott (1713-1788) who described the occurrence of scrotal cancer in chimney-sweeps in 1775 and traced it to the contamination of the skin by soot. Since then, various attempts have been made to induce cancer in experimental animals, but it was not until 1918 that the first successful induction of skin cancer in rabbits was announced by the Japanese team, Yamagiwa and Ichikawa (1918) who painted the ears of rabbits repeatedly with coal tar. After the carcinogenic nature of coal tar had been clearly established, many efforts were directed toward simplification of the technique of cancer induction and toward isolation and identification of active substances in coal tar. Great progress was made in this area of cancer research.

In 1936, the first successful induction of mammary cancer in experimental animals was reported. Maison and Coolen (1936) painted the skin of mice repeatedly with 3-methylcholanthrene (20-methylcholanthrene, MC) and benzpyrene (BP) and observed the appearance of mammary cancer, in addition to skin cancer, in 18% of the treated mice after 7 months. It was later disclosed that mammary cancer arose mainly in female mice (Engelbreth-Holm, 1941), and this pointed up the relationship of sex hormones and chemically induced mammary cancer.

While testing the toxicity of the insecticide, 2-acetamino-fluorene, (2-AAF) in rats, Wilson, DeEds and Cox (1941) unexpectedly
found that mammary cancer appeared, in addition to cancers of other organs, when the rats were fed on a diet containing 2-AAF for several weeks. This was the first evidence that mammary cancer could be induced by remote application of a chemical carcinogen. The results of Wilson et al (1941) were later confirmed by Bielschowsky (1944a). The above observations encouraged attempts to induce mammary cancer by other means. After administration of a small dose of MC (i.e. 2 mg. in 0.5 ml. olive oil) daily for several months by stomach tube, Shay, Aegerter, Gruenstein and Komarov (1949) observed a high incidence of mammary adenocarcinomas in female Wistar rats. Repeated intravenous injection of an oil emulsion of DMBA to female S-D rats also induced predominantly mammary tumors (Geyer, Bleisch, Bryant, Robbins, Saslaw and Stare, 1951).

By this time, it had become obvious that DMBA and MC selectively induced mammary tumors in rats. But the existing methods of tumor induction were very lengthy and risky because they required repeated treatment of the animals with the carcinogens for a prolonged period of time. A more simple and rapid method of tumor induction was urgently needed, and many investigators began to explore this problem. In 1959, Huggins and his associates (1959a) announced their successful attempt to induce mammary tumors in female S-D rats by a single oral dose of MC (20 to 75 mg.) and two years later, Huggins et al (1961a) further reported their successful induction of mammary tumors in S-D rats by a single intragastric instillation of DMBA (20 mg. in 1 ml. of sesame oil). A single oral dose of DMBA was very much superior to a single oral dose of MC as far as the induction of mammary tumors in
rats was concerned because a single oral dose of DMBA rapidly induced mammary tumors in all treated rats within 3 months whereas a single oral dose of MC could only evoke a small percentage of mammary tumors within 3 to 4 months. In a separate experiment, Huggins et al (1961b) also found that a single intravenous injection of an oil emulsion of DMBA (5 mg) was as effective as an oral dose of 20 mg. of DMBA in the induction of mammary tumors in S-D rats. The brilliant discoveries of Huggins and his associates had great influence in the field of experimental research on mammary tumor formation for Huggins' experimental model was widely used by other investigators in the study of the mechanism of mammary carcinogenesis.

In addition to the chemical carcinogens mentioned above, there are other agents, chemical and physical, that can evoke mammary cancer in certain strains of rats. Daily injection of estrone or subcutaneous implantation of estrone pellets induced mammary tumors in several strains of female rats after a long latent period (Geschickter, 1939; Noble, McEuen and Collip, 1940; MacKenzie, 1955; Cutts, 1966). Multiple pituitary grafts in rats and mice resulted in the induction of mammary tumors (Muhlbock and Boot, 1959; Welsch, Jenkins and Meites, 1970a). Lesions produced experimentally in the median eminence of the hypothalamus in mice and rats also caused the appearance of mammary tumors (Montemurro and Tob, 1967; Welsch et al, 1970b). A sublethal dose (400 r) of whole body X- or gamma irradiation was reported to evoke exclusively mammary tumors in female rats (Shellabarger, Cronkite, Bond and Lippincott, 1957; Huggins and Fukunishi, 1963a; Telles and Ward, 1969). Astatine 211, an alpha-emitting analogue of iodine, was
found to induce numerous malignant mammary tumors in female rats when injected intravenously at the dosage of 0.5 μg per kilogram of body weight (Durbin, Asling, Johnston, Farratt, Jaung, Williams and Hamilton, 1958). Recently, it was reported that prolonged immunosuppression resulted in the formation of mammary adenocarcinomas in dogs (Joseph, Melewicz and Morton, 1970). Two industrial chemicals, propylene imine and propane sulfone, were found to induce predominantly mammary tumors in addition to tumors of other types when they were administered orally to female rats for several weeks (Ulland, Finkelstein and Weisburger, Rice and Weisburger, 1971).

Mammary tumors induced by MC and DMBA were found consisting of mainly three histological types, namely, adenocarcinomas, fibroadenomas and adenomas (Daniel and Prichard, 1961, 1964a, 1964b; Middleton, 1965). The incidence of spontaneous mammary tumors was generally agreed to be very low in S-D rats (Bullock and Curtis, 1930; Dunning and Curtis, 1956; Thompson, Huseby, Fox, Davis and Hunt, 1961), but contradictory findings were reported by Davis, Stevenson and Busch (1956). Spontaneous mammary tumors usually occurred very late in the life span of the animals and were predominantly fibroadenomas and adenomas (Noble and Cutts, 1959).

II. THE TWO STAGE MECHANISM OF CARCINOGENESIS.

The concept of a two-stage mechanism of carcinogenesis originated from the experiments of Rous and Kidd (1941) and of Berenblum (1941). The studies of Rous and Kidd (1941) demonstrated that transitory papillomas induced on the ears of rabbits could be caused to reappear at, or about the same site by a further period of tarring,
by painting the ears with turpentine or by scarring the previously
tarred tissue. Berenblum's experiments (1941) showed that initial
application of a known carcinogenic tar to mouse skin in threshold
doses of tumor induction followed by subsequent local application of
croton oil or resin resulted in marked increase in tumor production. The
idea of carcinogenesis in stages was expanded by Tannenbaum (1944a) who
showed that caloric intake and high fat diet both had an effect on the
development of skin tumors in mice. He suggested that the carcinogenic
process studied by Rous and Kidd (1941), by Berenblum (1941) and by
himself could be divided into two distinct stages, namely the preneoplastic
or initiation stage and the neoplastic or developmental stage. However,
the two-stage theory of carcinogenesis was actually formulated by
Berenblum and Shubik (1947). According to this theory, a certain number
of normal cells are irreversibly transformed by the carcinogen into
latent tumor cells, (Initiating process). These latent tumor cells do
or do not develop into perceptible tumors, depending on the presence or
absence of suitable promoters. A promoter can not induce neoplastic
transformation by itself but will promote the development of latent
tumor cells to form visible tumors.

Since its formulation, the theory of two-stage carcinogenesis
has been tested in different experimental systems and its validity in
skin carcinogenesis confirmed by many investigators (Clayson, 1962).
There is also evidence that the two-stage mechanism operates in the
induction of tumors in other tissues, such as the thyroid (Bielschowsky,
1944b; Hall, 1948), the liver (Glinos, Bucher and Aub, 1951), and the
mammary gland (Dao and Sutherland, 1959b; Dao, 1962). In the case of
chemical induction of mammary tumors in the rat, the initiating process has been shown to be very rapid and irreversible (Dao, King and Gawlak, 1968; Dao, 1970), but mammary tumors only appear in the presence of ovarian hormones (Noble and Cutts, 1959; Dao, 1969a).

III. ACCUMULATION AND CLEARANCE OF POLYCYCLIC HYDROCARBONS IN MAMMARY TISSUE.

Dao et al (1959a) and Bock and Dao (1961) showed that MC selectively accumulated in the mammary tissue after oral administration. The levels of MC in the mammary tissue and fat were reported to be very much higher than in other tissues; but they decreased rapidly within the first 72 hours. A significant amount, however, remained in both mammary tissue and fat even at the end of the eighth day after administration. Using tritiated MC, Flesher and Sydnor (1960) found, on the contrary, that 24 hours after oral administration, the radioactivity in the liver and kidneys was higher than those of the mammary gland and fat. Goodall, McIntyre and Kennedy (1963) also found no specific localization of MC in the mammary gland. After intravenous injection of $^{14}\text{C}$-labelled DMBA, Lo (1965) reported no specific accumulation of DMBA in the mammary gland since the level of DMBA in the liver, kidneys and fat tended to be higher than that of the mammary gland. This finding was later confirmed by other investigators (Daniel, Pratt and Prichard, 1967; Flesher, 1967; Flesher and Sydnor, 1970). When tritiated DMBA and tritiated 7-hydroxymethyl-12-methylbenz(a)anthracene (7-OHM-12-MBA) were fed at the same dosage, the level of $\text{DMBA}^{-3}\text{H}$ was considerably higher than that of $\text{7-OHM-12-MBA}^{-3}\text{H}$ in the breast tissue 24 hours after administration; but at 72 hours after administration, the concentrations
of the two radioactive compounds in the breast tissue were only
slightly different, indicating that the rate of clearance of DMBA from
the breast tissue was much faster than that of 7-OHM-12-MBA. (Flesher,
technique, showed that DMBA appeared in the mammary gland shortly
after oral administration and reached peak level at 12 hours. After
that, the level declined rapidly but remained in measurable amounts
five days after administration. When female rats were treated with
DMBA and the mammary glands transplanted into recipients of the same
strain, Dao et al (1968) observed that the pattern of clearance of
DMBA from the grafted mammary gland appeared similar to that from the
mammary gland in situ for first 3 days, but thereafter the grafts seemed
to trap the carcinogen for as long as 30 days after transplantation,
while the carcinogen in the mammary gland in situ was no longer
measurable after 7 days.

All the above studies indicate that DMBA and MC both
accumulate in the mammary gland in appreciable amounts, not necessarily
the largest of any tissue, and the rate of clearance of these
carcinogens is very much slower in the mammary gland than in any other
tissues. The fact that chemical carcinogens remain in the mammary
gland for long periods of time led Dao and his associates (Dao, et al,
1959a; Bock and Dao, 1961) to suggest that the adipose tissue around
the mammary gland might serve as a depot for the carcinogens and thus
prolong the exposure and intensify the response of the glandular cells
to carcinogenic action of DMBA and MC. Recently, Janss and Moon (1970)
separated the parenchymal cells of the mammary tissue from the adipose
cells by enzymic treatment and found that the level of DMBA reached peak level in the adipose cells at 16 hours but at 6 hours in the parenchymal cells and parenchymal cell lipids. They suggested that the concentration of DMBA in the intercellular lipid of the parenchymal cells is of prime importance in the exposure of the parenchymal cells to the carcinogen.

The level of polycyclic hydrocarbons in the mammary gland is affected by several factors. Bock and Dao (1961) showed that the level of MC in mammary tissue is directly proportional to the dose given. A similar observation was reported by Flesher and Sydnor (1970). However, the dose-level correlation is true only within certain limits because increasing the dose of MC from 2 mg to 20 mg raised the tissue level from 4.2 μg/gm tissue to 33.3 μg/gm tissue; but increasing the dosage from 20 mg to 100 mg did not result in a corresponding increase in tissue level (Wieder, Thatcher and Shimkin, 1967). With multiple feeding experiments, the level of MC in the tissue was determined largely by the initial dose because repeating the initial dose several times did not result in increased tissue level (Bock and Dao, 1961; Wieder et al, 1967).

The nature of the hydrocarbons and the vehicles used also play a role in deciding the level of the carcinogen in the mammary tissue. DMBA, MC and BP rapidly accumulated in the mammary tissue, but only very little phenanthrene appeared in the mammary tissue after administration. The levels of DMBA and MC were not much different (Bock and Dao, 1961; Wieder et al, 1967), but the level of BP was much higher (Bock and Dao, 1961). The level of DMBA, however, was higher than
the level of its derivative, 7-OHM-12-MBA, in the mammary tissue when the two were fed at a 20 mg dose (Flesher and Sydnor, 1970). The difference could be due to the fact that the latter was less lipid-soluble than the former.

The hormonal status of the animal under study is one of the important factors that decide the level of carcinogen in the mammary tissue. In pregnant rats the level of MC was lower, but in hypophysectomized rats the level was higher than in normal, intact rats (Bock and Dao, 1961). The level of MC in the mammary tissue was higher in the females than in the males (Bock and Dao, 1961; Wieder et al, 1967).

There is no simple relationship between the level of different carcinogens in the mammary tissue and the ultimate carcinogenic response. For example, when DMBA and MC were administered at the same dosage, the level of these two carcinogens in the mammary tissue was not much different, but there was a great difference in the carcinogenic response (Bock and Dao, 1961; Wieder et al, 1967). However, to a limited extent, increase in carcinogenic response was proportional to the increase in level of the carcinogen in the mammary tissue. This statement is valid only when comparisons are made on the same carcinogen. Dao et al (1968) and Dao (1970) found that mammary tumor incidence in transplanted mammary grafts was directly proportional to the level of DMBA in the grafts. Flesher and Sydnor (1970) also found a similar relationship when different dosages of DMBA were administered intragastrically.
IV. METABOLISM OF DMBA AND THE PROXIMATE CARCINOGEN.

Liver is the main detoxifying organ of the body and is capable of metabolizing a great many foreign compounds. In general, harmful foreign compounds are metabolized to less harmful or inactive products, but there is evidence that certain substances, for example, 2-AAF, are metabolized to more active carcinogenic products (Miller and Miller, 1969).

The hepatic microsomal enzyme system known as benzpyrene hydroxylase, was shown to metabolize benzpyrene (BP) (Conney, Miller and Miller, 1957; Wattenberg and Leong, 1962). The same enzyme system was generally believed to metabolize other polycyclic hydrocarbons as well. Benzpyrene hydroxylase was shown to be present in the liver (Conney et al, 1957), in gastrointestinal tracts (Wattenberg, Leong and Strand, 1962), in lung and kidneys (Wattenberg and Leong, 1962; Dao and Yogo, 1964; Dao and Varela, 1966). The benzpyrene hydroxylase activity in the liver, lung, kidneys and gastrointestinal tracts was readily induced by treatment with a number of chemical compounds (Conney et al, 1957; Arcos, Conney and Buu-Hoi, 1961; Wattenberg et al, 1962; Dao and Yogo, 1964; Dao and Varela, 1966; Conney and Levin, 1966; Levin and Conney, 1967; Mullen, Juchaw and Fouts, 1962; Wattenberg, Page and Leong, 1968; Gelboin and Blackburn, 1964).

DMBA is metabolized in rat liver mainly by processes involving
ring hydroxylation and side-chain hydroxylation. The main products of side-chain hydroxylation are the isomeric monohydroxymethyl derivatives, that is, 7-hydroxymethyl-12-methylbenz(a)anthracene (7-OHM-12-MBA) and 12-hydroxymethyl-7-methylbenz(a)anthracene (12-OHM-7-MBA). (Boyland and Sims, 1965a, 1967a; Sims and Glover, 1968). 7-OHM-12-MBA is an active metabolite but it is less carcinogenic than the parent compound, DMBA, with regard to the induction of mammary tumors in rats. (Boyland, Sims and Huggins, 1965b; Flesher et al, 1967; Pataki and Huggins, 1967; Wheatley and Inglis, 1968; Flesher and Sydnor, 1970). A small dose of 12-OHM-7-MBA (20 mg.) was reported to be completely inactive (Boyland et al, 1965b), but a larger dose (30 mg.) was shown to be weakly mammary carcinogenic (Wheatley and Inglis, 1968). 7-12-Dihydroxy-7,12-dimethylbenz(a)anthracene and the ring hydroxylation metabolites were all regarded as inactive as far as the induction of mammary tumors in rats was concerned (Boyland and Sims, 1967a; Flesher et al, 1967; Wheatley and Inglis, 1968). However, 12-OHM-7-MBA had been shown to be capable of inducing sarcomas effectively in C57 mice. (Boyland et al, 1965b; Boyland and Sims, 1967b).

Unlike 2-AAF, metabolic studies with DMBA have failed to reveal any metabolite that is more carcinogenic than DMBA itself. The question of proximate carcinogen, therefore, remains speculative. However, biological evidence seems to favour the idea that DMBA is itself the proximate carcinogen that initiates the neoplastic transformation. Impairment of liver function by carbon tetrachloride which presumably reduced the metabolism of DMBA in the liver did not have any effect on the induction of mammary tumors by DMBA. (Kernohan,
Inglis and Wheatley, 1967; Tanaka and Dao, 1965). Dao et al. (1968) showed that tumors could occur in mammary grafts that were excised from the donors as soon as 10 minutes after an intravenous injection of DMBA and transplanted into non-treated recipients. In a recent study, Dao (1970) incubated mammary tissues in vitro in media containing different concentration of DMBA for 10 minutes at 4°C in the absence of light and transplanted the mammary tissues, after thorough washing, into non-treated recipients. He observed that tumors occurred in mammary grafts that had been incubated in media containing 5 mg. of DMBA or more. Dao and associates argued that if metabolism of DMBA was necessary for its carcinogenicity, the metabolism must occur in the mammary tissue. Since no metabolism of DMBA in the mammary tissue has been detected so far, they concluded that DMBA itself, rather than any of its metabolites, is the proximate compound that initiates the neoplastic transformation in the mammary gland.

V. DMBA-INDUCED ADRENAL NECROSIS AND ITS IMPLICATION ON MAMMARY CARCINOGENESIS.

Among the known chemical carcinogens, DMBA is the only one that can cause adrenal necrosis in female S-D rats. DMBA-induced adrenal necrosis is generally not fatal to the animals because regeneration of damaged tissue often takes place rapidly. Huggins and Morii (1961) observed massive necrosis in the inner zone of adrenal cortex. The lesion was restricted mainly to the zona fasciculata and zona reticularis of the cortex; the zona glomerulosa in the cortex and the medulla were spared and unaffected. In addition to inflicting damage on the adrenals, DMBA was found to cause necrosis in
the ovaries, but the lesion was not as severe as that found in the adrenals (Wong, Warner and Yang, 1962). Recently it was found that derivatives of 12-methylbenz(a)anthracene which possessed at position 7 of its molecule an alkyl, methoxymethyl, formyl or hydroxyalkyl group are active adrenocortocolytic agents (Huggins, Morii and Pataki, 1969).

7-OHM-12-MBA, a metabolite of DMBA, was found to be 6 times more active than DMBA with regard to the induction of adrenal necrosis in female rats, but its isomer, 12-OHM-7-MBA, was completely inactive (Boyland et al, 1965b). It was therefore suggested that DMBA was metabolized in the liver to an active metabolite which produced necrotic lesions in the adrenals. This hypothesis was tested in a series of experiments. Liver function was impaired by treatment with carbon tetrachloride, dl-ethionine and by partial hepatectomy so as to inhibit the metabolism of DMBA and it was found that these treatments effectively protected the adrenals against the damaging action of DMBA (Wheatley, Kernohan and Currie, 1966a; Wheatley, 1969). However, impairment of liver function by carbon tetrachloride, dl-ethionine and partial hepatectomy could not protect the adrenals from the adrenocortocolytic action of 7-OHM-12-MBA (Wheatley et al, 1966b; Wheatley, 1969). These findings supported the above hypothesis that DMBA-induced adrenal necrosis was caused by a metabolite which was likely to be 7-OHM-12-MBA.

Adrenal necrosis induced by DMBA and its metabolite, 7-OHM-12-MBA, could be inhibited by appropriate pretreatment of the animals with aromatic hydrocarbons, amines and certain drugs. MC, benzantracene, 6- aminochrysene, aminofluorene and 2-naphthylamine, given 24 hours before
the challenged dose of DMBA, were all effective protectors against
DMBA-induced adrenal necrosis (Dao and Tanka, 1963a; Huggings, Deuel
and Fukunishi, 1963b). As a matter of fact, many polynuclear hydro-
carbons, aromatic azo and ethylene derivatives were good protectors
of DMBA-induced adrenal necrosis at some dosage (Dao, 1964a; Huggins
deduced from these studies was that the most effective protectors were
flat, condensed aromatic compounds with 4 to 5 rings, and small doses of
these compounds were required to produce full protective effect (Huggins
and Fukunishi, 1964a; Dao, 1964a). However, the time interval between
the administration of protectors and the administration of the
challenger, DMBA, was of great importance because pretreatment with
aromatic protectors earlier than 48 hours before or later than 24 hours
after the administration of DMBA resulted in only partial inhibition
of adrenal necrosis or no inhibition at all (Dao, 1964a; Huggins and
Fukunishi, 1964a).

Certain drugs were found to possess the property of counter-
acting the adrenocorticolytic action of DMBA and its metabolite.
Metopirone (SU-4885), an 11β-hydroxylation inhibitor of adrenal
steroid synthesis, inhibited completely the induction of adrenal
necrosis by DMBA and 7-OH-12-MBA if it was given before the adreno-
corticolytic agents (Currie, Helfenstein and Young, 1962; Dao and
Tanaka, 1963; Wong and Warner, 1964; Wheatley et al, 1966b; Wheatley,
1968a; Jellinck, Garland and McRitchie, 1968). Recent studies further
indicated that metopirone not only protected maternal adrenals but also
protected fetal adrenals against the action of both DMBA and 7-OH-12-
MBA (Bird, Crawford, Currie and Stirling, 1970b). Other drugs such as SU-9055, and SKF 525-A, also protected against adrenal necrosis inflicted by DMBA and its metabolite, 7-OHM-12-MBA (Wong and Warner, 1964; Wheatley, 1968a, 1968b; Jellinck et al, 1968; Bird et al, 1970b). Spironolactone and ethylestrenol were recently added to the list of protective drugs (Kovacs and Somogyi, 1969; Somogyi and Kovacs, 1970a, 1970b). The protective effect of the drugs could be nullified by simultaneous administration of dl-ethionine (Wheatley, 1968a, 1968b; Symogyi and Kovacs, 1970b). Dl-ethionine itself was also a protector of adrenal necrosis induced by DMBA and 7-OHM-12-MBA if it was given 3 days before the administration of adrenocorticoalytic agents (Wheatley, 1969), but simultaneous administration of methionine and ethionine abolished the protective effect of the latter (Wheatley, 1969).

Pretreatment protection of DMBA and 7-OHM-12-MBA induced adrenal necrosis by various aromatic compounds and drugs was thought to be achieved through the stimulation of liver microsomal enzymes.

It is well established that most of the effective protectors of adrenal necrosis are also good inducers of benzpyrene hydroxylase in the liver (Arcos et al, 1961; Dao and Yogo, 1964; Conney and Levin, 1966). Pretreatment of rats with aromatic hydrocarbons increased the rate of metabolism of DMBA and its monohydroxymethyl derivatives (Boyland and Sims, 1965a; Boyland and Sims, 1967a). Further studies indicated that the main effect of pretreatment of rats with aromatic hydrocarbons was an alteration of the metabolic pathway of DMBA from side-chain hydroxylation to ring hydroxylation (Jellinck and Goudy, 1966, 1967; Jellinck and Smith, 1969). Ring hydroxylation of DMBA resulted in the
formation of inactive metabolites as far as the induction of adrenal necrosis was concerned. On the contrary, pretreatment of rats with metopirone and SU-9055 did not cause any alteration in the metabolism of DMBA in the liver, compared to animals that received DMBA alone (Jellinck et al., 1968; Jellinck and Garrett, 1969). No apparent change in corticosteroid synthesis, no inhibition of 11β or 21α hydroxylation of progesterone and deoxycorticosterone in the adrenals of rats pre-treated with metopirone and SU-9055 were observed (Jellinck et al., 1968).

The exact mechanism of the induction of adrenal necrosis by DMBA remains unknown, and the role of 7-OHM-12-MBA is being questioned for there was suggestion that 7-OHM-12-MBA might not be the final metabolite that inflicted damage on the adrenals. SKF 525-A, a drug that inhibited the drug-metabolizing enzymes in liver microsomes, completely inhibited the induction of adrenal necrosis by 7-OHM-12-MBA (Wheatley, 1968a, 1968b; Bird et al., 1970a). Both MC and Sudan III effectively protected the adrenals against necrosis induced by a standard dose (15 mg. p.o.) or overdose (30 mg. p.o. or 7.5 mg. i.v.) of 7-OHM-12-MBA (Wheatley and Sims, 1969). Spironolactone, a steroid drug, was recently shown to inhibit completely the induction of adrenal necrosis by 7-OHM-12-MBA (Somogyi and Kovacs, 1970b). All the above results indicated that 7-OHM-12-MBA as such could not inflict damage on the adrenals and further metabolism or conjugation was probably required to make it potentially adrenocorticoalytic. Recent studies on the embryopathic effects 7-OHM-12-MBA on maternal and fetal adrenals also provide evidence to suggest that 7-OHM-12-MBA was not likely to be ultimate metabolite of DMBA that caused damage on the adrenals of
female rats (Bird et al., 1970b).

Huggins and associates (Huggins and Morii, 1961; Huggins et al., 1964a) suggested that DMBA exerted its lytic action directly on the adrenal cortical cells. Kovacs and others (Howarth et al., 1968; Howarth et al., 1969), on the hand, claimed that adrenal necrosis occurred as a result of DMBA-inflicted damage on the capillary endothelial cells in the adrenal cortex. However, it is definite that in order for the adrenocortico-lytic action to take place a suitable hormonal environment must be present in the animal. For example, DMBA could not induce adrenal necrosis in immature rats (25-27 days old) and hypophysectomized rats were resistant to the induction of adrenal necrosis by DMBA unless ACTH was administered (Morii and Huggins, 1962). The corticosterone concentrations in the adrenals and in the plasma were significantly lowered 24 hours after DMBA treatment (Huggins et al., 1963c; Dao, et al., 1963b; Dale and Schutchfield, 1968), but in rats pretreated with aromatic hydrocarbons the level of corticosterone in the adrenals and in the plasma were similar to the level in rats without DMBA treatment (Huggins et al., 1963c). Dale and Schutchfield (1969) showed that there was a shift in the steroid biosynthetic pattern in the adrenal of DMBA-treated rats. During the first week of treatment, an increase in DOC (deoxycorticosterone) production from labelled progesterone and an overall decreased utilization of progesterone for all other adrenal corticosteroid syntheses were observed. However, during the second week after treatment, the biochemical changes were actually the reverse of those seen during the first week after treatment. From this information, the above authors concluded that DMBA caused partial
destruction of the hydroxylating enzymes in the adrenals which were required for the synthesis of adrenal steroids. Ascorbic acid was observed to decrease in adrenals after DMBA administration (Dao et al., 1963b). There was suggestive evidence that ascorbic acid might be involved in adrenal steroid synthesis (Hayano et al., 1956). It was also known that corticosteroids had the function of stabilizing lysosomal membrane and markedly inhibiting inflammatory response in small blood vessels after tissue injury (Weismann and Thomas, 1964). Therefore, under condition of reduced adrenal corticosteroid concentration, the lysosomal membrane might be rendered exceptionally susceptible to the action of DMBA or its metabolite. In vitro studies had shown that 7-OHM-12-MBA could release acid phosphatase from adrenal lysosomal preparations and it was suggested DMBA-induced adrenal necrosis might be due to the accumulation of an active metabolite of DMBA in the adrenal lysosomes, which eventually released the lytic enzymes to destroy the cells (Allison and Dingle, 1966).

The importance of adrenal function to mammary carcinogenesis is well established. The fact that a number of effective protectors against adrenal necrosis were also good protectors against cancer induced by DMBA makes this particular aspect of research of great interest to many cancer researchers. It was reported that multiple feedings of several aromatic hydrocarbons before DMBA administration resulted in varying degree of inhibition of mammary carcinogenesis (Huggins, Grand and Fukunishi, 1964b). 6-Aminochrysene seemed to be the most effective one in this regard. A single feeding of MC, BP, benzantracene (BA) and phenanthrene 48 hours prior to DMBA administration delayed the development of mammary tumors significantly (Dao, 1964b). Pretreatment with 1 mg. of MC 24 hours before DMBA administration was shown to reduce mammary tumor in-
cidence as well as delaying the appearance of the tumors (Wheatley, 1968d). Huggins and Pataki (1965) studied a series of aromatic azo derivatives in the prevention of adrenal injury and cancer induced by DMBA and found that the most effective protectors in this regard were Sudan III and compounds closely related to it. One interesting observation made during the above study was that the protective efficiency of these compounds ran parallel to their ability to induce menadione reductase in the liver. β-Naphthoflavone, a strong inducer of microsomal hydroxylase activity, markedly inhibited the occurrence of neoplastic lesions in the mammary gland when it was given prior to the carcinogen, DMBA (Wattenberg and Leong, 1968).

Pretreatment with SKF 525-A (10 mg/1 kgm. body weight) 1 hour before DMBA treatment increased mammary tumor incidence, but if the same drug was given 24 hours before the carcinogen, no enhancing effect could be detected (Wheatley, 1968c). Jull (1966) showed that injection of metopirone, starting 1/2 day before and continuing for 4 1/2 days after the administration of DMBA significantly reduced mammary tumor incidence in female rats. Spironolactone, a non toxic drug and a good protector of adrenal necrosis, decreased mammary tumor incidence and lengthened the latent period of the tumors induced by DMBA when the drug was given 4 days before and continued for 7 days after carcinogen administration (Kovacs and Somogyi, 1970). Estradiol, which promoted mammary carcinogenesis in female rats, was found to aggravate significantly the induction of adrenal necrosis by a suboptimal dose of DMBA when the hormone, 11.8 mg, was given orally twice daily for 5 consecutive days, beginning 2 days prior to DMBA administration (Somogyi and Kovacs, 1970a).
VI. FACTORS AFFECTING MAMMARY CARCINOGENESIS IN THE RAT.

Chemically induced mammary carcinogenesis in rats is affected by a number of factors, namely, genetic, age, nature of the carcinogen, dosage and route of administration of the carcinogen, hormones and nutrition. The hormonal and nutritional factors will be discussed in details in separate chapters because they are the subjects of interest in our studies. The other factors, however, will be discussed briefly in the following paragraphs.

It has been repeatedly demonstrated that different strains of rats exhibit different susceptibility toward the carcinogenic action of chemical carcinogens (Kim and Furth, 1960; Engelbart and Gericke, 1964; Boyland and Sydnor, 1962). The S-D rats were found to be the most vulnerable strain as far as the induction of mammary tumors was concerned (Huggins and Jensen, 1965; Gruenstein et al, 1966b).

Age is an important factor, too. With S-D rats, the best time to treat the animals with carcinogen was between 50-60 days of age since older or younger rats were refractory to the carcinogenic action of carcinogens (Dao and Sutherland, 1959; Huggins et al, 1961a; Meranze et al, 1969). Recently Dao (1970) showed that the age and the structure of mammary tissue at the time of carcinogen treatment was a key factor in deciding whether or not mammary tumor would be induced in the tissue.

DMBA and MC were the two most potent mammary carcinogens which induced predominantly mammary tumors when administered to female rats. Other carcinogens such as BP, AAF and several derivatives of aminophenan-threne were weak mammary carcinogens which required either repeated treatments or administration of large doses to be effective. (Huggins et al, 1959a, 1961b; Huggins and Yang, 1962; Dannerberg and Huggins, 1969).
Mammary tumors can be induced by a number of means such as intragastric instillation, intravenous injection, intraperitoneal injection and intracolonic injection of carcinogens (Huggins et al., 1961b; Huggins and Yang, 1962; Huggins and Fukunishi, 1963b). However, the first two methods are generally regarded as the best way to induce mammary tumors in rats.

VII. HORMONAL FACTORS IN MAMMARY CARCINOGENESIS IN RATS

Hormones are closely associated with the induction and development of mammary tumors in rats. This topic has been reviewed several times in the past (Noble and Cutts, 1959; Dao, 1964a, 1969a). However, much newer information has been obtained in recent years.

1. Involvement of Hormones in the Initiation of Mammary Tumors

It is well established that several pituitary hormones are involved in the normal development of mammary gland, and that hypophysectomy totally inhibits the induction of mammary cancer in rats by chemical carcinogens (Noble and Walters, 1954; Huggins, Grand and Brillantes, 1959b; Dao and Sutherland, 1959b). The pituitary gland in situ produces several tropic hormones which, in turn, control the secretion and function of other endocrine glands. Therefore, it is very difficult to assess correctly which of the pituitary hormones are involved in the mammary carcinogenic process in rats, simply based on the information obtained with hypophysectomy alone. The secretion and release of pituitary hormones are, on the other hand, regulated by the hypothalamus. Klaiber, Gruenstein and Shimkin (1969) reported that hypothalamic lesions alone did not result in the appearance of mammary tumors in female S-D rats; but Welsch, Nagasawa and Meites (1970b) observed 53% mammary tumors incidence in hypothalamic-lesioned female rats in contrast to 19% mammary tumor incidence
in non-lesioned controls. The results of Welsch et al were in agreement with earlier findings of Montemurro and Toh (1967) in mice. The apparent discrepancy in the preceding results could be due to the fact that Klaiber et al used virgin, young adult female rats in their experiments whereas Welsch et al used multiparous adult female rats.

Hypothalamic lesions placed before administration of DMBA significantly inhibited mammary tumor induction in female rats (Clemens et al, 1968; Klaiber et al, 1969). Welsch et al (1970b) observed that the plasma prolactin level in hypothalamic-lesioned rats was significantly higher than that of non-lesioned controls. It was shown that pituitary grafts isolated from hypothalamic control secreted predominantly prolactin and a small amount of few other pituitary hormones (Meites and Nicoll, 1966). Transplantation of pituitary grafts into intact female rats subsequently treated with DMBA markedly inhibited mammary tumor induction (Welsch et al, 1968). Pretreatment of intact female rats with 'Enovid', a contraceptive composed of norethynodrel-mestranol, 10 to 25 days before the administration of DMBA, significantly reduced the incidence and the yield of mammary tumors as compared to the tumor incidence induced by DMBA alone (Weisburger et al, 1968; Welsch and Meites, 1969a). Recent in vitro studies indicated that insulin and prolactin could induce lobulo-alveolar growth of rat mammary gland (Juergens, Stockdale, Topper and Elies, 1965; Dilley and Nandi, 1968; Turkington, 1968) so it is probable that as a result of hypothalamic lesions and pituitary grafting, the increased prolactin secretion stimulates lobulo-alveolar growth of the mammary gland and renders the mammary gland refractory to the carcinogenic action of the
carcinogen. This assumption is in agreement with the earlier findings of Dao et al that pregnancy and lactation at the time of carcinogen treatment greatly inhibited the induction of mammary tumors (Dao et al, 1960). Recently Welsch et al (1968) found that injection of progesterone (4 mg/day) for 40 consecutive days starting when the rats were 30 days old markedly inhibited subsequent induction of mammary tumors by DMBA. Similar results were obtained by Moon (1969) who found that a single pregnancy, with or without a subsequent nursing period prior to DMBA feeding, resulted in decreased mammary tumor incidence and increased latent period. A further increase in the number of pregnancies and lactation periods did not greatly influence the incidence of mammary tumor nor the number of mammary tumors per rat.

In the case of spontaneous mammary tumorigenesis, multiple pituitary grafts significantly enhanced the incidence in female S-D rats compared to non-grafted controls (Welsh, et al, 1970a). However, transplantation of pituitary grafts onto ovariectomized rats did not provoke any mammary tumor (Welsch et al, 1968) and hypothalamic lesions placed in ovariectomized rats did not show any effect on chemically-induced mammary carcinogenesis (Klaiber et al, 1969). The preceding results indicate that prolactin alone has little or no effect on mammary carcinogenesis, but prolactin and estrogen could act synergistically.

In the early 1940's, Bielschowsky (1944a, 1947) observed that, out of 36 female and 41 male rats fed on a ration containing 2-AAF, 69% of the females and 7% of the males had mammary tumors. When rats were castrated, 1 out of 11 spayed females had mammary tumors and none of
the castrated males had breast neoplasm. These results indicated to him that the presence of ovaries was a factor of great importance in the development of mammary tumors in rats. Similar findings were obtained by Shay, Gruenstein and Harris (1952) when they treated male and female rats with MC; thus demonstrating the close relationship between sex and chemical induction of mammary tumors. Later, in 1959, it was found by two groups of investigators that ovariectomy performed one week before or immediately after the administration of MC or DMBA reduced mammary tumor incidence but did not prevent completely the formation of mammary tumors in female rats (Huggins, Briziarelli and Sutton, 1959a; Huggins et al, 1959b; Dao and Sutherland, 1959b; Dao, 1962a). These results were confirmed later by other investigators (Sydnor and Cockrell, 1963; Heimann, Heuson and Coune, 1968). Huggins et al (1959a, 1959b) found that the inhibitory effect of ovariectomy could be nullifying by daily injection of 1 µg of estradiol-17ß or 4 mg. of progesterone. However, extending the above studies, Dao (1962a) in 1962 reported that ovariectomy performed 30 days before the administration of MC could totally inhibit the induction of mammary tumors by this carcinogen. This interesting and important experiment was repeated by other investigators using DMBA as the tumor inducing agent and similar results were obtained (Talwalker, Meites and Mizuno, 1964; Welsch et al, 1968; Terenius, 1971; Beuving, 1969). The above results strongly suggest that the presence of sufficient amounts of estrogen in the animal body at the time of carcinogen treatment is essential for the induction of mammary tumors in female rats.

Male rats are generally highly resistant to the induction of mammary tumors by chemical carcinogens. Some investigators reported
complete failure in mammary tumor induction in male rats (Dao and Sutherland, 1959b; Howell, 1960), whereas others (Huggins et al, 1969b; Dao, 1964a; Huggins and Grand, 1966; Shay et al, 1959; Gruenstein et al, 1966a; Bielschowsky, 1944a; 1947) were only able to induce mammary tumors in a small percentage of the treated male rats. However, when ovaries were grafted to intact male rats which had been treated with MC, mammary tumors developed in 14% of the male rats receiving ovarian grafts; but when ovaries were grafted to castrated, MC-treated male rats, mammary tumors increased markedly from 14% to 66% (Dao and Griener, 1961). The studies with male rats provide additional evidence that the presence of estrogen is conducive to the initiation of mammary tumors by chemical carcinogens.

Simultaneous administration of estrogen and chemical carcinogen, on the other hand, did not produce consistent results. Geyer et al (1953) reported that simultaneous injection of estradiol or diethylstilbestrol (0.6 mg) and DMBA accelerated the appearance of mammary tumors as well as increased the incidence. Huggins et al (1959b), on the contrary, found that daily injection 10 μg of estradiol in conjunction with MC feeding reduced mammary tumor incidence and delayed its appearance. Others (Cantarow et al, 1948; Schaller and Carnes, 1958) found that administration of estrogen side by side with chemical carcinogen did not affect carcinogenesis. Injection of estriol, 100 μg per 100 gm body weight daily for 12 days beginning 3 days from the administration of DMBA significantly reduced the incidence and delayed the appearance of mammary tumors compared to control rats treated DMBA alone (Terenius, 1971).

The preceding studies indicate that estrogen might have dual
effects on mammary carcinogenesis and this property of estrogen was demonstrated by Huggins et al (1959a) and by Kim, Furth and Yannopoulos (1963). Daily injection of estradiol-17β in small amounts (0.1-3µg) stimulated, while daily injection of large amounts of estradiol-17β (20-30 µg) inhibited the development of mammary tumors induced by MC. How estrogen brings about these dual effects on mammary carcinogenesis is still unknown, but Kim et al (1963) observed that changes in mammary tumor size occurred concurrently with changes in plasma prolactin level. With large doses of estradiol, the pituitary enlarged and contained much prolactin but the plasma prolactin level decreased. Using a radioimmunoassay technique, Chen and Meites (1970) demonstrated recently that daily administration of 0.1 to 500 µg of estradiol benzoate for 6 days increased the plasma prolactin level by several fold, but small doses of 0.1-5 µg estradiol benzoate seemed to be more effective in increasing plasma prolactin level than the larger doses of estradiol benzoate. Larger doses of estradiol, however, seemed to inhibit the release of prolactin in the pituitary. The results of Chen and Meites are therefore in accordance with early observations of Kim et al (1963).

Although estrogen can promote the synthesis and release of pituitary prolactin both by a direct action on the pituitary and via the hypothalamus (NicolL and Meites, 1962; Meites and NicolL, 1966), in view of all the previous findings, it is most likely that prolactin and estrogen work synergistically to facilitate the induction of mammary tumors by chemical carcinogens.
2. Involvement of Hormones in the Development, Growth and Maintenance of Mammary Tumors.

Noble and Collip (1941) were perhaps the first to demonstrate clearly that the growth and maintenance of induced mammary tumors were dependent on hormones. They induced mammary tumors in female hooded rats by estrone pellets implanted subcutaneously. After withdrawing the estrone pellets or the source of exogenous supply of estrogen, they observed complete regression of well-established tumors in four rats under study.

The pituitary gland is generally regarded as the centre of control of all endocrine activities, but pituitary functions are in turn controlled by the hypothalamus. In 1959, Huggins et al (1959b) and Dao and Sunderland (1959b) both showed that hypophysectomy of tumor-bearing rats caused a profound decrease in tumor size in all animals under study. Similar results were later reported by Sterenthal et al (1963). However, two groups of investigators obtained different results after hypophysectomy from those described above. Kim and Furth (1960) found that only 85% of the mammary tumors induced by MC regressed after hypophysectomy. Daniel and Prichard (1963a) reported that mammary tumors in 19 out of 20 rats regressed after hypophysectomy, but the tumor in the remaining one remained unaffected.

Sectioning of the pituitary stalk is one way of disrupting pituitary functions and this was shown by Daniel and Prichard (1963a) to cause complete regression of mammary tumors in approximately 2/3 of a group of tumor-bearing rats, tumors in the remaining 1/3 being unaffected.
Hypothalamic lesions, or more specifically, lesions of the median eminence of the hypothalamus, greatly enhanced mammary carcinogenesis when they were placed after DMBA administration, in contrast to their inhibitory effect when placed before the administration of DMBA (Clemens et al, 1968; Klaiber et al, 1969; Welsch et al, 1969b). The effect of pituitary stalk section and lesions of median eminence of hypothalamus on pituitary functions were the same qualitatively, that is, they increased the secretion of prolactin and reduced the secretion of all other pituitary hormones (McCann and Dhariwal, 1966; Meites, 1966). Welsch et al (1969b) went on to study the effect of lesions in other areas in the hypothalamus and found that, unlike lesions of the median eminence, preoptic and amygdaloid lesions placed in tumor-bearing rats caused marked regression of existing tumors.

Ovariectomy has no uniform effect on mammary tumors in rats. Out of 8 tumor-bearing rats which were ovariectomized subsequently, Huggins et al (1959a, 1959b) observed that tumors in 7 of the rats showed considerable regression in size, but tumors in the remaining one continued to grow. Dao and Sutherland (1959b) reported complete regression of mammary tumors in all rats under study after bilateral ovariectomy. Daniel and Prichard (1963b), on the other hand, reported that only half of a group of well-established tumors showed sign of complete regression after ovariectomy; the remaining half either showed partial regression or no regression at all. The results of Daniel and Prichard were confirmed by other investigators (Young, Cowan and Sutherland, 1963; Teller et al, 1966a, 1969; Gropper and Shimkin, 1967). The above findings indicate that the presence of two types of mammary
tumors in rats, the hormone-dependent and hormone-independent tumors.

Young et al (1963) found that most of the tumors that regressed after ovariectomy could be reactivated to grow again by administration of estradiol or a combination of estradiol and progestosterone. Kim and Furth (1960) demonstrated that grafting of functional mammotropic tumors to ovariectomized tumor-bearing rats caused not only the resumption of tumor growth but also the appearance of new tumors. Daily injection of progesterone (4 mg) nullified the inhibitory effect of ovariectomy and accelerated the appearance of mammary tumors (Huggins et al, 1959a).

Administration of progesterone alone to female rats has not been found to induce mammary tumors (Huggins et al, 1959a; Grunstein et al, 1964; Jabara, 1967), so it is generally considered that progesterone acts as a co-carcinogen in mammary carcinogenesis (Huggins et al, 1962; Kim, 1965).

Early in 1948, Cantarow (1948) found that progesterone greatly enhanced mammary carcinogenesis induced in rats by 2-AAF. Later, in 1959, progesterone was found to enhance mammary carcinogenesis induced by MC and DMBA (Huggins et al, 1959a). Huggins' results were confirmed by other investigators (Gruenstein et al, 1964; Jabara, 1967). Huggins and Yang (1962) reported that injection of progesterone into intact rats for 30 days, beginning 15 days after DMBA administration, resulted in accelerated appearance of mammary cancers. Jabara (1967) extended this study and found that progesterone significantly increased mammary tumor incidence regardless of whether it was given 2 days before or 15 days after DMBA feeding. This is in accordance with the early findings
that pregnancy occurring after carcinogen treatment markedly increased the tumor incidence and shortened the latent period (Dao and Sunderland, 1959b; Dao et al., 1960; Huggins et al., 1962; McCormick and Moon, 1965). If pregnancy occurred after the appearance of palpable mammary tumors, it caused the tumors to grow faster and enhanced the occurrence of new tumors (McCormick and Moon, 1965). Similar results were obtained by others when progesterone was injected into tumor-bearing rats (Huggins et al., 1962; Jabara, 1967). The promoting effect of progesterone could be nullified, to a great extent, by concurrent treatment with a large dose of estradiol. Huggins et al. (1962) observed that only half of the DMBA-treated rats which subsequently received daily injection of estradiol (20 μg) and progesterone (4 mg.) for 30 days beginning at the age of 65 days had mammary tumors at the end of 6 months' observatory period, whereas their control mates which received DMBA and no hormone treatment had all succumbed to mammary cancers. In ovariectomized rats, concurrent treatment of DMBA and prolactin or STH also resulted in increased mammary tumor incidence compared to rats fed DMBA alone (Talwalker et al., 1964).

Dao and Sutherland (1959b) reported that parturition and lactation caused regression of MC-induced, well-established mammary tumors in all animals under study, but McCormick and Moon (1965, 1967) reported that parturition and lactation did not have uniform effect on well-established mammary tumors induced by DMBA. They found that a majority of the tumors regressed with the occurrence of parturition and lactation, but a small proportion of the tumors either continued to grow or remained static. However, if lactation were combined with
ovariectomy, regression of all well-established mammary tumors was then observed. Administration of 2-Br-α-ergocryptine, a suppressor of lactation and nidation, for 6 weeks to mammary tumor-bearing rats was reported by Heuson et al (1970) to cause regression of established tumors and inhibition of the formation of new tumors. Ergocornine and iproniazid injection for 15 days into tumor-bearing rats also suppressed the growth of established tumors and the formation of new tumors (Nagasawa and Meites, 1970). Radioimmunoassay indicated that ergocornine significantly reduced plasma and pituitary prolactin level but iproniazid had little or no effect on prolactin level.

Huggins et al (1959b) noticed that adrenalectomy alone failed to inhibit the induction of mammary tumors. This result was later confirmed by others (Shay et al, 1960; Sydnor and Cockrell, 1963), but Daniel and Prichard (1967), on the other hand, observed that adrenalectomy significantly encouraged the induction of mammary tumors in S-D rats. If adrenalectomy and ovariectomy were performed together, the induction of mammary tumors was completely inhibited (Sydnor and Cockrell, 1963; Durbin et al, 1966).

Gruenstein et al (1966c) observed that when adrenalectomy was performed after surgical removal of the first tumor, it appeared to inhibit the occurrence of additional tumors but not sufficiently to be statistically significant. Sterenthal et al (1963) reported that adrenalectomy and ovariectomy, if performed together within 10–20 days after the tumors were first detected, caused regression of mammary tumors in all rats. Estrogen administration reactivated the growth of mammary tumors which regressed after adrenalectomy–ovariectomy but
after hypophysectomy. On the contrary, Daniel and Prichard (1967) found that adrenalectomy performed in tumor-bearing rats stimulated the growth of all existing tumors. This finding was, however, in line with the observations of Kim et al (1960) and Kim (1965).

Injection of hydrocortisone in conjunction with MC treatment reduced the number of tumors in intact rats but did not influence the total incidence of mammary tumors (Sydnor and Cockrell, 1963). Injection of corticosterone 1/2 day before and 4 1/2 days after DMBA administration significantly reduced mammary tumor incidence in rats (Jull, 1966).

The resistance of male rats to the induction of mammary tumors suggests that the male sex hormones, the androgens, may be inhibitory to the development of mammary neoplasia. Pretreatment of female rats at the age of 1–2 days old with a single dose of testosterone was found to reduce the incidence of mammary tumors induced by DMBA subsequently (Kovacs, 1965). Briziarelli (1965), on the other hand, found that injection of testosterone propionate 6 times each week for 30 days, beginning 10–20 days after DMBA administration, significantly retarded the appearance of mammary tumors and lengthened the latent period, but if the same testosterone treatment was given 30 days after DMBA administration, mammary tumor incidence was greatly increased. Huggins et al (1959a) observed regression of mammary tumors to varying degrees occurred in 14 out of 17 rats injected daily with 1–2 mg. of dihydrotestosterone, but the tumors in the remaining three rats continued to grow. Similar observation was made by Young et al (1965) using testosterone and dihydrotestosterone. Gruenstein et al (1966c) found
in rats, whose palpable tumor was surgically removed, that testosterone propionate treatment significantly decreased the occurrence of additional tumors.

However, evidence so far accumulated indicated that androgens had no uniform effect on the growth of mammary tumors induced by chemical carcinogens. Tumors regressed after androgen treatment could be reactivated by the administration of suitable dose of estradiol and progesterone. It was, therefore, speculated that the effect of androgens was probably mediated through the pituitary.

Bielschowsky and Hall (1953) showed that, in the rat thyroidectomy had an inhibitory effect on the induction of mammary tumors with 2-acetylaminofluorene. Similar results were obtained by Jull and Huggins (1960) in thyroidectomized rats treated with MC. The latter workers found that rats treated with 500 μg of L-thyroxine per day exhibited enhanced development of MC-induced mammary cancer, but in rats treated daily with 1000 μg of thyroxine the mammary tumor incidence was significantly inhibited. The inhibitory effect of hypothyroidism on mammary tumor induction was also demonstrated by Heffenstein et al (1962) who treated rats with propylthiouracil (PTU), a chemical which produced hypothyroid functions in animals. However, the dose of thyroxine used by Jull and Huggins (1960) was 250-500 times greater than the estimated rate of secretion of thyroxine in that strain of rats (Newman and Moon, 1966). Extending this study, Newman and Moon (1968) found that administration of 2.5 μg of thyroxin per 100 gm body weight daily, beginning 10 days before MC feeding and continued for 7 months thereafter, did not alter the response of the rats to the carcinogenic
effect of MC. However, chronic administration of PTU, a chemical hypothyroid agent, during the same period significantly inhibited mammary tumor induction by MC. On the contrary, Eskin et al (1968) reported that hypothyroidism produced chemically 10 days before the administration of DMBA greatly accelerated the induction of mammary neoplasia in female rats but if hypothyroidism was produced after the treatment of DMBA, no significant effect on mammary carcinogenesis could be seen.

Jull and Huggins (1960) ascribed the inhibitory effect of thyroidectomy on mammary tumor induction to smaller caloric intake in the thyroidectomized rats compared to intact animals. Newman and Moon (1968), on the other hand, thought that the observed effect was due principally to the lack of growth promoting stimulus of thyroid hormone on the neoplastic cells.

VIII. EFFECTS OF DIETARY FAT ON CHEMICAL CARCINOGENESIS IN MICE AND RATS

The research on nutrition and cancer had been reviewed by Tannenbaum and Silverstone (1953, 1957), by Tannenbaum (1959) and by Clayson (1962). Studies in the past on the effect of dietary fat on chemical carcinogenesis had been complicated by the problem of caloric intake. Increased caloric intake was found to parallel increased tumor incidence in mice (Tannenbaum, 1940, 1942a, 1945; Visscher, Ball, Barnes and Silversten, 1942; White, Burroughs, Kelly and Heston, 1944). However, it was later found that not all tumors were equally sensitive to the effect of increased caloric intake (White, 1961).

Reports on the effect of dietary proteins on carcinogenesis in mice and rats are contradictory to each other and they will not be reviewed here because in our experiments the diets have approximately the same content of proteins. Thus, dietary protein is not an important factor in our studies.
1. Influences of Dietary Fat on Various Types of Tumors

In a pioneer investigation, Watson and Mellanby (1930) discovered in 1930 that feeding mice with diets containing 12.5 to 25% butter fat caused a definite increase in the incidence of skin tumors induced by tarring. Later in 1939, Baumann and Rusch (1939a) showed, in their study on the induction of skin tumors by ultraviolet light, that skin tumors were produced in greater numbers and at earlier times in mice fed a high fat (cottonseed oil) diet than in mice fed on commercial diet. Extending this study, Baumann and his group further demonstrated that high fat diet not only increased the incidence of skin tumors induced by tarring and by ultraviolet light but also enhanced the incidence of skin tumors induced by painting and injection of carcinogenic agents such as benzpyrene (BP), 3-methylcholanthrene (MC) and dibenzanthracene (DBA) (Baumann, Jacobi and Rusch, 1939b; Jacobi and Baumann, 1940). These findings were later confirmed by other investigators (Lavik and Baumann, 1941, 1943; Tannenbaum, 1942b, 1944; Boutwell, Brush and Rusch, 1949).

Studies on the effect of a fat-enriched diet on the formation of tumors were extended to other types of neoplasms, both spontaneous and induced. Utilizing spontaneous mammary carcinoma of the mouse, Tannenbaum (1942b) reported that high fat diet significantly increased the incidence of spontaneous mammary carcinomas and shortened the latent period of the tumors. These results were later confirmed by Silverstone and Tannenbaum (1950) who further showed that the incidence of spontaneous mammary carcinomas increased and the latent period of the
tumors decreased with increasing proportion of dietary fat. However, the increase in tumor incidence was not arithmetically proportional to the content of fat in the diet. It seemed that the effect of dietary fat on spontaneous mammary carcinomas in the mouse was quite consistent, but this was not the case when spontaneous mammary tumors in rats were studied. Lavik and Baumann (1943) found that high fat diet had no effect on spontaneous mammary tumors in rats. Davis et al (1956), on the other hand, observed that spontaneous mammary tumor incidence in female S-D rats was higher in those fed on fat-enriched diet than in those fed on control diet. Benson et al (1956) also noticed an increase in the incidence of mammary fibroadenomas in female S-D rats fed on high fat diet.

In the case of induced mammary tumors in rats, Dunning et al (1949) found that high fat diet significantly increased the tumor incidence and shortened the latent period of mammary tumors induced by implantation of diethylstilbestrol pellets. The incidence of mammary tumors induced by feeding a diet containing 0.03% AAF was found to be significantly increased when 15 to 30% by weight of lard were added to the diet (Engel and Copeland, 1951). Gammal et al (1967) reported that a high fat diet containing 20% corn oil significantly increased the incidence and shortened the latent period of mammary tumors induced by a single intragastric instillation of 5 mg. of DMBA, compared to a low fat diet containing 0.5% corn oil. The results of Gammal et al were recently confirmed by Carroll and Khor (1970).

Dietary fat seemed to have a variety of effects on tumor formation. In a study concerned with the dependence of tumor formation
on the composition of calorie-restricted diet, Tannenbaum (1945b) incidentally discovered that spontaneous hepatomas in C3H male mice responded to high fat diet in a manner similar to skin tumors and mammary tumors. In a later experiment, Silverstone and Tannenbaum (1951) showed that increasing the fat content of the diet from 2 to 20% resulted in an enhancement of the rate of formation of the spontaneous benign hepatomas in C3H mice. However, in rats the situation was quite different and contradictory results may be found in the literature. It was reported that the addition of 20% fat to a synthetic diet containing p-dimethylaminoazobenzene resulted in accelerated formation of hepatomas as compared with a similar diet containing little fat (Kline, Miller, Rusch and Baumann, 1946). Silverstone (1948) on the other hand, reported that fat had no effect on p-dimethylaminoazo-benzene-induced hepatoma and Engel and Copeland (1951) found that 2-AAF-induced liver tumor did not appear to be influenced by changes in the level of dietary fat. Littman, Taguchi and Mosbach (1966) reported in 1966 that a cholesterol-free, fat-free diet retarded the growth of Novikoff hepatoma in mice. The nature of the fat appeared to be a decisive factor because hydrogenated coconut oil was found to inhibit the formation of induced hepatoma whereas corn oil acted as promoter for the same tumor (Miller, Kline, Rusch and Baumann, 1944a, 1944b).

High fat diet was found to have no effect on primary lung adenoma of the mouse and induced sarcoma (Tannenbaum, 1942b), and to have an inhibitory effect on ocular orbit tumors and ear-duct tumors (Engel and Copeland, 1951). The development of pituitary adenoma in mice treated with radioactive iodine was also reported to be enhanced by a high fat ration (Silberberg and Silberberg, 1953).
2. Mode of Action of Dietary Fat on Tumor Formation

Although there is ample evidence in the literature to support the observation that dietary fat enhances the development of skin and mammary tumors in mice and in rats, there has been very little study concerning the mechanism of action of dietary fat on these two types of tumors. In an experiment designed to study the potency of different fat fractions in stimulating skin tumor production, Lavik and Baumann (1941) found that the tumor-promoting activity of fat resided in the fatty acid fraction. Ethyl laurate was as effective as natural glycerides but glycerol and the unsaponifiable fraction had little or no activity. When heated treated fat was added to the diet at the level of 10% by weight, tumor incidence was markedly increased. The tumor incidence was 71% in the group fed a diet containing heat-treated fat as compared to 31% in the group fed a diet containing untreated fat. The peroxide number of heat-treated fat was 2 and that of the untreated fat was 4.8. In the case of spontaneous mammary tumors in the rat, different results were reported. Nolen, Alexander and Artman (1967) found that, when rats were fed diets containing 15% used frying fats (i.e. soybean oil, cottonseed oil and lard), the tumor incidence was 20-40% lower than those fed on diets containing fresh soybean oil. The peroxide values were all high in the fried fats. However, Poling, Eagle, Rice, Durand and Fisher (1970) found that there was little difference in the incidence of spontaneous mammary and non-mammary tumors when rats were fed diets containing either heat-treated or untreated fats. The peroxide values were however reported to be low and little changed by the heating
procedure. On the other hand, Sugai, Witting, Tsuchiyama and Kummerow (1962) reported that heat-treated corn oil acted in synergism with AAF and enhanced its carcinogenicity which resulted in considerable increase in the incidence of liver tumors, ear-duct tumors and mammary tumors as compared with the relatively low tumor incidence when fresh, untreated corn oil was used. The carcinogenicity of heated and oxidized fats has been the subject of extensive studies and a review of this area was recently written by Artman (1969) and O'gara, Stewart, Brown and Hueper (1969).

According to the two-stage theory of carcinogenesis formulated by Berenblum and Shubik (1947), the process can be divided into two distinct stages, referred to as the initiation stage and the promotion stage. Lavik and Baumann (1941) noticed that, in mice, high fat diet fed only after the painting of MC had ceased could still increase skin tumor incidence by about 30% more than the control. However, the effectiveness of high fat diet was greater when it was fed for the entire experiment. Using a slightly different technique, Tannenbaum (1944) showed that high fat diet fed only after the application of BP had ceased and before the appearance of any tumor in all groups, was as effective in enhancing tumor incidence as high fat diet fed throughout the experiment. This observation led the above investigator to conclude that high fat diet exerted its action mainly on the developmental stage of the carcinogenic process. Carroll and Khor (1970) induced mammary cancers in rats by a single intragastric instillation of DMBA and found that high fat diet fed 24 hours after the administration of carcinogen increased the tumor incidence and tumor yields to the same extent as
high fat diet fed throughout the entire experiment. This finding led to
the conclusion that high fat diet enhanced mammary carcinogenesis in rats
by affecting mainly the developmental stage of the carcinogenic process.
It is interesting to note that high fat diet affects the carcinogenic
process occurring in two different tissues, the skin and the mammary
gland, in a similar fashion.

Attempts to explain the promoting effect of high fat diet on
tumor formation have been complicated by the fact that high fat diet
usually possesses a greater caloric value than low fat diet. Lavik and
Baumann (1941) suggested that the promoting effect of high fat diet could
be due partly to a local effect on the skin of the mice and partly to an
increase in caloric intake. Boutwell et al (1949) believed that the
greater net energy of high fat diet was alone sufficient to account for
its enhancing effect. Tannenbaum (1944b, 1959), on the other hand,
argued that the enhancing effect of high fat diet on tumor formation was
due to some specific action of fat which was independent of general
caloric intake. It was shown by Dunning et al (1949) in a pair-fed
experiment, that the rats on both low and high fat diets had isocaloric
intake, but the mammary tumor yield was higher and the latent period
was shorter in the group consuming high fat diet than in the group
consuming low fat diet. Similar results were also obtained by Silver-
stone and Tannenbaum (1950) in mice which received isocaloric low and
high fat diets. In the experiments of Gemmal et al (1967) and of
Carroll and Khor (1970) the body weight of rats on low and high fat
diets was little different and the mammary tumor incidence was again
higher and the latent period was shorted in the group fed on high fat
diet than in the group fed low fat diet. Even at a particular
restricted caloric intake, a diet high in fat content was less in-
hibitory than a diet that had little fat in it (Tannenbaum, 1945b).
Another observation that supported the view of Tannenbaum was that high
fat diet had no effect on the incidence of lung tumor (Tannenbaum,
1942b). Lung tumor should be augmented if the effect of high fat diet
was due to increased caloric intake.

A dual action of dietary fat was suggested, that is, solvent
action and co-carcinogenic action (Tannenbaum, 1942b). For solvent
action, Tannenbaum (1959) explained that high fat diet might increase
the fat content of the tissue and thus alter the storage and transfer
of the carcinogen. He regarded the co-carcinogenic action of fat as an
independent effect of fat on the developing tumor cell. The solvent
action of fat has received considerable support from many investigators
who measured the level of carcinogen in the tissues (Dao et al, 1959;
Gammal et al, 1968; Shay et al, 1950; Wieder et al, 1967), but the
exact nature of the co-carcinogenic action of dietary fat has not been
clearly established.

IX. DISTRIBUTION OF LABELLED ESTRADIOL IN VARIOUS ANIMAL TISSUES AFTER
INJECTION.

In 1962, Jensen and Jacobson (1962) studied the uptake of
tritiated estradiol in several estrogen non-responsive tissues (i.e.
liver, kidney, muscle and blood) and estrogen responsive tissues (uterus
and vagina) in young immature rats. They noticed that radioactivity
reached a maximum very rapidly in the estrogen non-responsive tissues and
thereafter declined very rapidly, whereas radioactivity in the uterus and
vagina reached a maximum slowly and the radioactivity was retained for a much longer period. The results of Jensen and Jacobson were confirmed by Roy, Mahesh and Greenblatt (1964). Parallel experiments were carried out in mice with similar results (Stone, 1963). Eisenfeld and Axelrod (1965; 1966) extended the above study using mature intact and ovariectomized female rats and found that tritiated estradiol-17β selectively accumulated in the anterior pituitary, uterus, vagina and hypothalamus. Rat mammary tissue was found by Sander (1968) to accumulate more radioactivity per unit wet weight than muscle and fat, after an injection of tritiated estradiol-17β. King et al (1965) reported that DMBA-induced mammary adenocarcinomas selectively accumulated tritiated estradiol when the animal was injected with the labelled estrogen. The finding of King et al was confirmed by Mobbs (1966), but progesterone was not taken up or retained by the mammary adenocarcinomas to the same extent as estradiol (Mobbs, 1968a). In fact estradiol-17β accounted for over 90% of the radioactivity in the target tissues, but only 40% or less of the radioactivity in the liver could be attributed to unchanged tritiated estradiol (Jensen and Jacobson, 1962; King et al, 1966).

When norethynodrel was given before the injection of tritiated estradiol, the concentration of the radioactivity in the anterior pituitary, uterus, vagina and hypothalamus was markedly diminished (Eisenfeld and Axelrod, 1965). Reduced accumulation of tritiated estradiol was also reported for uterus and vagina in animals treated with 17 α-ethyl-19-nortestosterone (Stone, 1964) and in vagina and pituitary in animals treated with clomiphene (Roy et al, 1964).
Administration of cold estradiol, estrone and estriol, either before or after the injection of tritiated estradiol, decreased the accumulation of radioactivity in the anterior pituitary, uterus, vagina, hypothalamus and mammary tissue but not in the heart, muscle, cerebrum, fat and plasma (Eisenfeld and Axelrod, 1965; 1966; Sander, 1968). High doses of progesterone, testosterone and hydrocortisone did not reduce the concentration of radioactivity in the target tissues (i.e. anterior pituitary, uterus, vagina and hypothalamus) and ovariectomy did not alter the pattern of estradiol uptake (Eisenfeld and Axelrod, 1966). The observations that estradiol was selectively taken up by the anterior pituitary, uterus, vagina, hypothalamus, mammary tissue and DMBA-induced mammary adenocarcinomas and that estrogen antagonists or analogues competitively inhibited the uptake of labelled estradiol in the target tissues suggested the presence of specific binding sites in these tissues.

Various attempts were made to locate the binding sites in the target cells. Using cell fractionation and autoradiographic techniques, King et al (1965b, 1966b) found that most of tritiated estradiol taken up by the anterior pituitary was localized in the nucleus. In the uterus, a major portion of the radioactivity taken up by uterine epithelial cells was present in the cytoplasm (King et al, 1966a), but a considerable amount of radioactivity was also associated with the nucleus (King et al, 1966b, 1968; Mobbs, 1968b; Stumpf, 1969). Nuclear concentration of estradiol appeared to be characteristic in all target cells (Mobbs, 1966b; Stumpf, 1969; King et al, 1968). The studies of King et al (1966a) and of Mobbs (1968b) indicated that there was an
exchange of tritiated estradiol between the nucleus and cytoplasm in the uterine epithelial cells, but the exact nature of this exchange process is still unknown.

The estrogen receptors in the uterus and DMBA-induced mammary adenocarcinomas have been isolated and partially characterized. Toft et al (1966, 1967) and Jensen et al (1969) found that the estrogen present in the soluble fraction after homogenization was bound to a macromolecule which consisted, at least in part, of protein and which had a sedimentation constant of 9.5 S. The nuclear receptor was thought to be a protein of high molecular weight which may be a component of the chromatin (King and Gordon, 1967; 1968). The nuclear receptor protein has been isolated from uterus and DMBA-induced mammary adenocarcinomas and found to have a sedimentation constant of about 5 S (Puca et al, 1968, King et al, 1969). The cytoplasmic and nuclear estrogen receptors are thought to be related because they are both precipitated by protamine sulphate (King et al, 1969).
MATERIALS AND METHODS

I. MATERIALS

A. Animals.

Virgin female Sprague-Dawley rats were obtained from Sprague-Dawley Co., Inc., Madison, Wisconsin, U.S.A. either at the 21-22 or 50-51 days of age.

B. Chemicals, Solvents and Reagents.

7,12-dimethylbenz(o)anthracene (DMBA) [Eastman Organic Chemicals] was purified by recrystallization from methanol and water before use (Carroll and Khor, 1970). The purified DMBA was made up in sesame oil, U.S.P. in a volumetric flask to a concentration of 5 mg. of DMBA per 0.25 ml. of sesame oil.

DMBA fat emulsions (Schurr, 1969) containing 5 mg. of DMBA per ml. of emulsion were kindly donated by Dr. Paul E. Schurr of the Upjohn Company, Kalamazoo, Michigan, U.S.A.

Corn oil, lard, butter and olive oil were purchased locally. Other fats and oils were kindly supplied by Procter and Gamble Ltd., Hamilton, Ontario.

Estradiol-6,7-3H (sp. act. 40 c/μM) and progesterone-1,2-3H (sp. act. 50.3 c/μM) were obtained from New England Nuclear, Boston, Mass. Estradiol-4-14C (sp. act. 40 mc/μM) was obtained from Schwartz Bioresearch, Inc., Orangeburg, N.Y. Labelled estradiol and progesterone were dissolved in 5% ethanolic saline for injection.

FPO, 2,5-diphenyloxazole and dimethyl-POPOP, 1,2-bis[2-(4-
methyl-5-phenyloxazolyl)-] benzene were both scintillation grade and were obtained from Packard Instrument Company, Inc., Downers Grove, Illinois.

Liquid scintillation solution was made up as follows: 7 gm. of PPO, 0.3 gm. of dimethyl-POPOP and 120 gm. of naphthalene crystals were dissolved in 1 litre of dioxane. The solution was stored in a brown bottle.

Acetic anhydride-sulphuric acid reagent was prepared fresh before use. 20 ml. of acetic anhydride, analytical reagent, [British Drug Houses (Canada) Ltd., Toronto, Ontario.] was placed in a round-bottomed flask and chilled in ice. When cool, 1 ml. of concentrated sulphuric acid was added dropwise, with shaking and mixing. The reagent was kept in ice at all times before and during use.

Digitonin, certified reagent, was obtained from Fisher Scientific Company, Toronto, Ontario.

All other chemicals and solvents used in this study were of reagent grade. Ethanol was redistilled before use.

II. METHODS
A. Long-term feeding experiments

On arrival, weanling female rats, 21-22 days old, were randomly divided into groups, each having equal number of rats. They were housed two to a cage in a well-ventilated, temperature-controlled room (76±2° F) with 12 hours of light and darkness. On the day of their arrival, they were put on semisynthetic test diets, the composition of which is shown in Appendix I. Semisynthetic test diets
were prepared weekly and stored in covered containers in a cold room at 4°C. The food cups were filled three times a week and water was given ad libitum. The animals were weighed weekly and their body weight recorded.

At 50 days of age, all animals were given a single oral dose of 5 mg. of DMBA in 0.25 ml. sesame oil by stomach tube (no. 8 French catheter) unless otherwise mentioned. The administration of DMBA was performed between 1.30 p.m. and 3.00 p.m. To minimize any possible effect the test diets might have on the absorption of the carcinogen, all animals were temporarily switched to Purina laboratory chow (Ralston Purina Co., St. Louis, Missouri) two days before and continued on this diet until one day after DMBA administration. Thereafter, all animals were returned to their respective semisynthetic test diets as scheduled. All experiments were terminated four months after the treatment with carcinogen.

Beginning one month after the administration of DMBA, the rats were checked regularly for palpable mammary tumors. The positions of the tumors were recorded and this record was used as a guide at autopsy to differentiate the palpable tumors from the non-palpable ones.

At autopsy, all rats were killed by chloroform. Tumors and suspected tissues were dissected out and preserved in 10% buffered neutral formalin until sectioning. All sections were stained with hematoxylin and eosin and the slides were examined by light microscopy.

B. Biochemical Analysis

1. Determination of the Apparent Digestibility of Dietary Fats.

The apparent digestibility of different dietary fats was
determined on groups of 6 rats during the course of one of the dietary experiments. The animals had been on diet about 3 months when these experiments were carried out. Feces were collected daily for 5 consecutive days and pooled for each group on a daily basis.

Fecal lipids were extracted according to the method of Friedner and Moberg (1967). Two gm of dry feces were ground in a mortar and transferred to 100 ml centrifuge tube with 10 ml of 96% ethanol. To the mixture was added 4-6 drops of concentrated HCl and 2 ml of water and the lipids were extracted with 2 portions of 40 ml petroleum ether (30-60° C). The combined extract was transferred to a weighed beaker and dried, first under nitrogen and then overnight in a desicator over P₂O₅. The Coefficient of Apparent Digestibility was calculated as

\[
\frac{\text{fat ingested} - \text{fat excreted}}{\text{fat ingested}} \times 100.
\]

2. **Determination of Fatty Acid Composition of Fats and Oils**

The fats (50-100 mg) were transmethylated by refluxing for 2 hours with a mixture prepared by adding 1 volume of acetyl chloride dropwise to 9 volumes of reagent grade methanol (Fieser, 1955) which was chilled in ice. The methyl esters of fatty acids were then extracted with petroleum ether (30-60° C), and analyzed by gas-liquid chromatography in a Beckman GC-45 with hydrogen flame detector, using a column of 15% EGSS-X on Chromosorb P. The results were quantitated by integration and were checked with NIH standard mixture of fatty acid methyl esters.
3. The Distribution of Radioactivity in Rat Tissues after Injection of Tritiated Steroid Hormones.

In these experiments, two groups of female rats were used. One group was purchased at 21-22 days of age and fed on high fat diet (20% corn oil) and low fat diet (5% corn oil) until they were 50-60 days of age. During the 10-day period, rats at diestrus were injected with 1 million counts of estradiol-17β-6,7-3H (0.1 μg) in 0.5 ml. 5% ethanolic saline. The other group was purchased at 50-51 days of age and fed on high and low fat diets until 90-100 days of age. During the 10-day period, rats at diestrus were injected with 2 millions counts of estradiol-17β-6,7-3H (0.2 μg) in 1 ml. of 5% ethanolic saline. At 1/2, 1 and 2 hours after injection, the animals were sacrificed by decapitation. Blood samples were collected in heparinized tubes. The pituitary, adrenals, ovaries, uterus, liver and mammary glands were dissected out, blotted dry and weighed. These tissues were frozen and stored at -14°C until analyzed.

The frozen tissues were thawed at room temperature. In the case of pituitary, ovaries, adrenals, uterus and mammary glands, the tissues were homogenized and extracted at the same time with chloroform-methanol (2:1). The homogenates were transferred to centrifuge tubes and centrifuged at 2000 r.p.m. for 20 minutes. The whole supernatant fraction from each sample was decanted into liquid scintillation counting vials except in the case of the mammary glands where an aliquot of the supernatant fraction was taken for counting. The liver was homogenized and extracted with 20 volumes of chloroform-methanol (2:1) overnight. The chloroform-methanol extract was filtered through
Whatman No. 1 filter paper with extensive washing with chloroform-
methanol (2:1) and these repeated washings removed virtually all the
radioactivity from the residues and the filter paper. The filtrate
was concentrated in vacuo on rotary evaporator to a small volume and an
aliquot of it was taken for counting. The blood sample, however, was
dispersed in methanol and then a calculated volume of chloroform was
added to the methanol so that the final proportion of chloroform and
methanol was 2 to 1. The extraction was allowed to go overnight. There-
after the chloroform-methanol extract was filtered, concentrated and
an aliquot was taken as described for the liver. In all cases,
the solvent in the counting vials was dried under nitrogen, and 15 ml.
of scintillation solution was added to each vial. The radioactivity
was determined on Packard Tri-carb Liquid Scintillation Spectro-
meter, Model 3375. The counting efficiency for tritium was 34% and
quenching was corrected by method of external standardization.

At the beginning of these experiments, acetone, diethyl ether
and chloroform-methanol (2:1) were used to extract the radioactivity
from the ovaries, adrenals and uterus and it was found that chloroform-
methanol extraction gave the best results. Therefore this solvent
mixture was used exclusively in our studies for extracting labelled
steroids from tissues.

4. Metabolism of Estradiol-4-\(^{14}\)C in the Liver in vivo

Female S-D rats were purchased at 50-51 days of age and fed
on high and low fat diets until they were 90-100 days of age. During
this period, vaginal smears were done daily and rats at diestrus were
injected intravenously (through the tail vein) with 1 million counts
of estradiol-4-$^{14}$C (0.2 µg) in 0.5 ml. of 5% ethanolic saline while the
animals were under light ether anaesthesia. One hour after the in-
jection, the rats were sacrificed by decapitation. The liver was
dissected out, blotted dry, weighed and stored frozen at -14°C until
analysis.

The liver was thawed, minced, homogenized and extracted with
20 volumes of chloroform-methanol (2:1) overnight. The chloroform-
methanol extract was filtered through Whatman No. 1 filter paper with
repeated washings. The actual volume of the extract was measured and
an aliquot was taken for counting (Total radioactivity). The remainder
of the extract was transferred to a separating funnel and 0.6% sodium
chloride solution equivalent to 1/4 of the total volume of chloroform-
methanol extract, was added. The contents of the funnel were mixed and
allowed to separate into two layers, the lower chloroform layer and the
upper methanol-water layer. The upper layer was extracted once again with
15 ml. of chloroform. The chloroform portions were combined. The
volumes of the chloroform fraction and methanol-water fraction were
measured and an aliquot was taken from each fraction for counting. The
radioactivity in each fraction was expressed as a percentage of the total
radioactivity.

5. **Analysis of Plasma Lipids.**

Female rats were bought at 50–51 days of age and were fed on
high fat diet (20% corn oil) and low fat diet (5% corn oil) until they
were 90 to 100 days of age. Blood was taken by heart puncture between
1:30 p.m. and 3.00 p.m. while the rats were under light ether
anaesthesia. Plasma was collected after centrifugation at 2000 r.p.m. for 30 minutes and stored at -14°C.

Thawed plasma of 2-3 rats was pooled in each determination and extracted with 20 vol. of chloroform–methanol (2:1) overnight at room temperature. However, it was necessary to add the plasma to methanol first and chloroform was added later so that the final proportion of chloroform and methanol was 2 to 1. The next day, the chloroform–methanol extract was filtered with repeated washings with the same solvent. The filtrate was then transferred to a separating funnel and a small volume of 0.6% sodium chloride solution was added. The contents were mixed and allowed to separate into layers. The lower organic phase was collected for determination of total lipids, phospholipids, neutral lipids, cholesterol and triglycerides in plasma.

Total lipids were determined gravimetrically after first evaporating the solvent to dryness on a rotary evaporator and then overnight in desiccator over phosphorus pentoxide. Phospholipids and neutral lipids were also determined gravimetrically after separation on acid-treated Florisil column (Carroll, 1962). Cholesterol was determined from the neutral lipid fraction according to the method of Sperry and Webb (1950) as modified and used routinely in our laboratory. Triglyceride level in plasma was also determined from the neutral lipid fraction according to the method of Van Handel and Zilvermit (1957). However, the triglycerides were hydrolyzed for 30 minutes instead of 15 minutes which was used by above investigators.

Plasma free fatty acid was determined according to the method of Goss and Lein (1967) with some modifications. To 1 ml. of fresh plasma
in a glass-stoppered centrifuge tube was added 5 ml. of extraction mixture consisting of 20 vol. of isopropyl alcohol, 5 vol. of heptane and 1 vol. of 1.0 N sulphuric acid. After the addition of extraction mixture, the plasma was vigorously shaken for 10 minutes. Three ml. of heptane were added followed by the addition of 2 ml. of freshly boiled distilled water. The tube was again shaken vigorously for another 10 minutes. Two 3 ml. portion of the upper heptane fraction were withdrawn for titration.

0.1 N sodium ethoxide prepared by dissolving 2.3 gm. of metallic sodium in 1 litre of absolute ethanol was used as base in the titration. However, this solution was diluted before use in small quantities to 0.016 N by the addition of absolute ethanol. 0.5 ml. of 0.01% phenolphthalein solution was used as indicator and the end point, from colourless to pink, was determined visually.

The titration was performed using a "Agla" micrometer syringe with a long, thin, curved needle. The syringe was fitted to a micrometer so that the volume of the base could be precisely controlled. During the titration, a stream of nitrogen was bubbled through the titrated heptane solution in order to agitate the solution and to prevent absorption of carbon dioxide from the air. A standard solution of palmitic acid in heptane, treated similarly as the unknown, was titrated with each group of unknown. A blank was obtained by taking 1 ml. freshly boiled distilled water through both the extraction and titration procedure. The titration value of the blank was substracted from the titration value of the standard and the unknown. The concentration of plasma free fatty acid was calculated as follows:
<table>
<thead>
<tr>
<th>Value of Standard (μEq./litre)</th>
<th>Value of Unknown (μEq./litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Titration value</td>
<td>Titration value</td>
</tr>
</tbody>
</table>

III. STATISTICAL METHODS

1. Student's t test was used for comparison between mean values.

2. Chi-Square Test (with Yate's correction) was used for comparison of tumor incidence between groups.

3. A P value of less than 0.05 is regarded as significant and where a more significant difference is obtained the P value is indicated in brackets.
EXPERIMENTAL RESULTS

Effect of Different Levels of Dietary Fat on Mammary Tumor Incidence

This series of experiments was actually carried out together with another series which was planned to study the effect of different types of dietary fat on mammary tumor incidence. To minimize variations due to the use of different lots of animals at different times, the rats were allotted into groups of 10 each and were put on semisynthetic diets containing 0.5%, 5%, 10% and 20% by weight of corn oil. The complete set of experiments was repeated twice to bring the total to 30 rats each. The diet containing 10% corn oil, however, was not included in the first trial, and 20 rats were therefore allotted to this diet in a subsequent experiment.

At 50 days of age, the rats were treated with a single oral dose of 5 mg. of DMBA by stomach tube and a switch from semisynthetic diets to commercial chow diet was followed as described in 'Methods'. Thereafter the rats were returned to their respective semisynthetic diets which were continued until the end of the experiments.

The cumulative palpable mammary tumor incidence in female rats fed on diets containing different levels of corn oil is shown in Fig. 1. The palpable tumor incidence indicates the percentage of rats with tumor palpated before the autopsy. The groups on 10% and 20% corn oil diets had considerably higher palpable tumor incidence than either of the group on 0.5% or 5% corn oil diet. However, a significant difference (p<0.05) was found only between the group on 10% corn oil diet and the
Fig. 1. Effect of diets containing different levels of corn oil on the cumulative palpable mammary tumor incidence in female rats treated with DMBA.
group on 0.5% corn oil diet. There was not much difference in palpable tumor incidence between the group on 10% corn oil diet and the group on 20% corn oil diet and likewise, the groups on 0.5% and 5% corn oil diets had similar palpable tumor incidence.

The complete results at autopsy are summarized in Tables I and II. There was no significant difference in mean body weight among the four groups at autopsy or at any time during the course of this experiment. At autopsy, the group on 10% corn oil diet had significantly (P<0.05) higher total tumor incidence than the group on 0.5% corn oil diet, but not the group on 5% corn oil diet. The difference in total tumor incidence between the group on 20% corn oil diet and either of the group on 0.5% or 5% corn oil diet was not large enough to be significant at 5% level. The group on 10% corn oil diet had significantly (P<0.025) more tumors per rat than either of the group on 0.5% or 5% corn oil diet, but did not have significantly more tumors per tumor-bearing rat or a significantly shorter latent period than the groups on either 0.5% or 5% corn oil diet. The group on 20% corn oil diet, besides having higher tumor incidence, also had more tumors per rat, more tumors per tumor-bearing rat and had a shorter latent period, but unfortunately the differences were not large enough to be significant at 5% level statistically.

Table II indicates that, irrespective of the level of corn oil in the diet, majority of the mammary tumors induced after a single administration of DMBA were adenocarcinomas. The main difference among the four groups of rats consuming on diets containing different levels of corn oil resided in the rate of appearance and number of palpable
Table I. Mammary tumor incidence following administration of DMBA to rats on semisynthetic diets containing different levels of corn oil.

<table>
<thead>
<tr>
<th>Corn oil diet*</th>
<th>Body Weight (gm.)**</th>
<th>Tumor Incidence†</th>
<th>No. of Tumors per Rat**</th>
<th>No. of Tumors per Tumor-bearing Rat**</th>
<th>Latent Period (days)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td>Total</td>
<td>Palpable</td>
<td>Total</td>
</tr>
<tr>
<td>20%</td>
<td>41.2±0.9</td>
<td>246.2±4.5</td>
<td>90.0</td>
<td>86.6</td>
<td>3.7±0.6</td>
</tr>
<tr>
<td>10%</td>
<td>38.8±0.3</td>
<td>239.9±6.6</td>
<td>93.3</td>
<td>93.3</td>
<td>4.0±0.5</td>
</tr>
<tr>
<td>5%</td>
<td>41.5±1.1</td>
<td>238.9±4.2</td>
<td>76.6</td>
<td>73.3</td>
<td>2.3±0.4</td>
</tr>
<tr>
<td>0.5%</td>
<td>42.9±0.5</td>
<td>238.6±2.5</td>
<td>70.0</td>
<td>70.0</td>
<td>2.5±0.4</td>
</tr>
</tbody>
</table>

*Thirty rats per group

**Mean ± S.E.M.

†Per cent of rats with mammary tumors.
Table II. Incidence of different types of mammary tumors in rats on semisynthetic diets containing different levels of corn oil.

<table>
<thead>
<tr>
<th>Corn oil diet</th>
<th>Adenocarcinomas</th>
<th>Fibroadenomas</th>
<th>Adenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Palpable</td>
<td>Nonpalpable</td>
<td>Total</td>
</tr>
<tr>
<td>20%</td>
<td>76</td>
<td>29</td>
<td>105</td>
</tr>
<tr>
<td>10%</td>
<td>85</td>
<td>25</td>
<td>110</td>
</tr>
<tr>
<td>5%</td>
<td>49</td>
<td>17</td>
<td>66</td>
</tr>
<tr>
<td>0.5%</td>
<td>45</td>
<td>21</td>
<td>66</td>
</tr>
</tbody>
</table>
Fig. 2. Effect of diets containing different levels of corn oil on the rate of appearance of palpable mammary tumors in female rats treated with DMBA.
tumors (Fig. 2), since there was no difference in the number of tumors that were not palpated before autopsy.

Effect of Different Types of Dietary Fat on Mammary Tumor Incidence

As mentioned in the previous experiment, this series of experiments was carried out initially with 10 animals in each group and the same set of experiments was repeated twice to bring the total to 30 rats per group. In the first feeding trial, the diet containing 20% lard was not included, and therefore in a subsequent experiment 20 rats were allotted to this diet.

The administration of DMBA and the change of diet immediately before and after the treatment of carcinogen were followed as mentioned in the previous experiments. Thereafter, all groups were returned to their respective semisynthetic diets as scheduled and the experiments were terminated 4 months after the treatment of carcinogen. The cumulative palpable mammary tumor incidence in rats fed different types of fats and oils at the level of 20% by weight of the diet is shown in Fig. 3.

Among the groups on diets containing saturated fats and oils (i.e. coconut oil, butter, tallow and lard), the groups on coconut oil and butter diets gave palpable mammary tumor incidence comparable to the group on corn oil diet, but the cumulative mammary tumor incidence of the groups fed on tallow and lard diets was somewhat lower than that of the group on corn oil diet for most of the experimental period (Fig. 3a). However, among the groups fed on unsaturated fats and oils (i.e. olive oil, rapeseed oil, cottonseed oil, corn oil, soybean oil and sunflower-seed oil), the palpable mammary tumor incidence was not much different (Fig. 3, b and c) except the group on rapeseed oil which had a palpable
Fig. 3. Effect of different dietary fats and oils fed at the level of 20% by weight of the diet on the cumulative palpable mammary tumor incidence in female rats treated with DMBA. The high corn oil group was included in Figs. 3a, 3b and 3c for the sake of comparison.
mammary tumor incidence lower than the rest of the groups on unsaturated fats and oils (Fig. 3b). In fact, the palpable tumor incidence of the group on rapeseed oil diet was similar to that of the group on tallow diet (Fig. 3, a and c).

The final results at autopsy are summarized in Tables III and IV. In Tables III and IV, the groups are arranged, from top to bottom, approximately in the order of increasing unsaturation of dietary fats and oils. The fatty acid composition of these fats and oils are shown in Table V. The animals grew somewhat differently on diets containing different fats and oils and the lowest final body weight was seen in the group fed on tallow and rapeseed oil diets which differed significantly ($P<0.05$) only from the groups fed on butter, cottonseed oil and soybean oil diets (Table III). Tallow and rapeseed oil had the lowest coefficient of apparent digestibility among the different fats and oils used in this study (Table III).

The tumor incidence of the different dietary groups was somewhat variable. A significant difference in total tumor incidence was found only between the groups on rapeseed oil and soybean oil diets and a significant difference in palpable tumor incidence was found between the groups on tallow and cottonseed oil diets (Table III). With the exception of the group on rapeseed oil diet, all the groups on diets containing unsaturated fats and oils had more mammary tumors per rat and more mammary tumors per tumor-bearing rat than the groups on diets containing saturated fats and oils. In fact, the groups on cottonseed oil and sunflowerseed oil diets had significantly more total tumors per rat and more tumors per tumor-bearing rat than the groups on coconut oil and tallow diets. The group on sunflowerseed oil diet also had
Table III. Mammary tumor incidence following administration of DMBA to rats on diets containing different fats and oils at the level of 20% by weight of the diet.

<table>
<thead>
<tr>
<th>Diet*</th>
<th>Coeff. of Apparent Digestibility (%)</th>
<th>Body Weight (gm.)**</th>
<th>Tumor Incidence† Total Palpable</th>
<th>No. of Tumors per Rat** Total Palpable</th>
<th>No. of Tumors per Tumor-bearing Rat**</th>
<th>Latent Period** (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% Coconut Oil</td>
<td>96.8</td>
<td>42.1±0.9</td>
<td>247.1±3.1</td>
<td>96.6</td>
<td>86.6</td>
<td>2.4±0.33</td>
</tr>
<tr>
<td>20% Butter</td>
<td>94.2</td>
<td>41.6±0.7</td>
<td>252.2±2.7</td>
<td>86.6</td>
<td>83.3</td>
<td>2.9±0.40</td>
</tr>
<tr>
<td>20% Tallow</td>
<td>87.6</td>
<td>40.4±0.8</td>
<td>239.5±3.7</td>
<td>80.0</td>
<td>70.0</td>
<td>2.4±0.45</td>
</tr>
<tr>
<td>20% Lard</td>
<td>93.9</td>
<td>39.1±0.6</td>
<td>243.3±5.3</td>
<td>93.3</td>
<td>86.6</td>
<td>3.2±0.46</td>
</tr>
<tr>
<td>20% Olive Oil</td>
<td>98.1</td>
<td>42.1±0.8</td>
<td>241.0±2.6</td>
<td>86.6</td>
<td>80.0</td>
<td>3.9±0.46</td>
</tr>
<tr>
<td>20% Rapeseed Oil</td>
<td>92.5</td>
<td>42.5±0.6</td>
<td>237.5±5.1</td>
<td>76.6</td>
<td>73.3</td>
<td>2.2±0.44</td>
</tr>
<tr>
<td>20% Cottonseed Oil</td>
<td>96.6</td>
<td>42.9±0.7</td>
<td>258.2±2.7</td>
<td>93.3</td>
<td>93.3</td>
<td>4.2±0.71</td>
</tr>
<tr>
<td>20% Corn Oil</td>
<td>97.4</td>
<td>41.2±0.9</td>
<td>246.2±4.5</td>
<td>90.0</td>
<td>86.6</td>
<td>3.7±0.58</td>
</tr>
<tr>
<td>20% Soybean Oil</td>
<td>97.8</td>
<td>42.6±0.6</td>
<td>252.5±1.1</td>
<td>100.0</td>
<td>86.6</td>
<td>3.4±0.45</td>
</tr>
<tr>
<td>20% Sunflower-seed Oil</td>
<td>96.9</td>
<td>41.5±0.8</td>
<td>248.4±4.8</td>
<td>96.6</td>
<td>86.6</td>
<td>4.3±0.46</td>
</tr>
</tbody>
</table>

* Thirty rats in each group. ** Mean ± S.E.M. † Per cent of rats with mammary tumors.
Table IV. Incidence of different types of mammary tumors following administration of DMBA to rats on diets containing different fats and oils.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Adenocarcinomas</th>
<th></th>
<th>Fibroadenomas</th>
<th></th>
<th>Adenomas</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Palpable</td>
<td>Nonpalpable</td>
<td>Total</td>
<td>Palpable</td>
<td>Nonpalpable</td>
<td>Total</td>
</tr>
<tr>
<td>20% Coconut Oil</td>
<td>51</td>
<td>18</td>
<td>69</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>20% Butter</td>
<td>59</td>
<td>20</td>
<td>79</td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>20% Tallow</td>
<td>53</td>
<td>17</td>
<td>70</td>
<td>2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>20% Lard</td>
<td>78</td>
<td>13</td>
<td>91</td>
<td>3</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>20% Olive Oil</td>
<td>85</td>
<td>24</td>
<td>109</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>20% Rapeseed Oil</td>
<td>41</td>
<td>21</td>
<td>62</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>20% Cottonseed Oil</td>
<td>87</td>
<td>35</td>
<td>122</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>20% Corn Oil</td>
<td>76</td>
<td>29</td>
<td>105</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>20% Soybean Oil</td>
<td>76</td>
<td>25</td>
<td>101</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>20% Sunflower-seed Oil</td>
<td>86</td>
<td>38</td>
<td>124</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>
Table V. Fatty acid composition of fats and oils (wt. %)*

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>Shorthand designation</th>
<th>Coconut Oil</th>
<th>Butter</th>
<th>Tallow</th>
<th>Lard</th>
<th>Olive Oil</th>
<th>Rapeseed Oil</th>
<th>Cottonseed Oil</th>
<th>Corn Oil</th>
<th>Soybean Oil</th>
<th>Sunflowerseed Oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caprylic</td>
<td>8:0</td>
<td>5.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Capric</td>
<td>10:0</td>
<td>6.6</td>
<td>1.0</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lauric</td>
<td>12:0</td>
<td>53.5</td>
<td>2.5</td>
<td>0.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Myristic</td>
<td>14:0</td>
<td>18.4</td>
<td>10.5</td>
<td>3.4</td>
<td>1.5</td>
<td>-</td>
<td>0.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Palmitic</td>
<td>16:0</td>
<td>7.9</td>
<td>31.0</td>
<td>25.5</td>
<td>25.5</td>
<td>11.3</td>
<td>3.0</td>
<td>19.6</td>
<td>10.1</td>
<td>10.4</td>
<td>5.5</td>
</tr>
<tr>
<td>Palmitoleic</td>
<td>16:1</td>
<td>-</td>
<td>2.5</td>
<td>2.8</td>
<td>2.5</td>
<td>0.5</td>
<td>0.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stearic</td>
<td>18:0</td>
<td>1.6</td>
<td>13.6</td>
<td>24.9</td>
<td>15.0</td>
<td>2.2</td>
<td>1.3</td>
<td>2.2</td>
<td>1.6</td>
<td>3.8</td>
<td>4.6</td>
</tr>
<tr>
<td>Oleic</td>
<td>18:1</td>
<td>5.0</td>
<td>29.9</td>
<td>35.7</td>
<td>44.5</td>
<td>78.5</td>
<td>23.3</td>
<td>18.2</td>
<td>31.4</td>
<td>24.2</td>
<td>14.7</td>
</tr>
<tr>
<td>Linoleic</td>
<td>18:2</td>
<td>0.8</td>
<td>1.8</td>
<td>1.6</td>
<td>9.3</td>
<td>7.2</td>
<td>16.0</td>
<td>59.2</td>
<td>56.3</td>
<td>53.5</td>
<td>75.1</td>
</tr>
<tr>
<td>Linolenic</td>
<td>18:3</td>
<td>-</td>
<td>0.6</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>8.0</td>
<td>-</td>
<td>0.4</td>
<td>7.8</td>
<td>-</td>
</tr>
<tr>
<td>Eicosanoic</td>
<td>20:1</td>
<td>-</td>
<td>-</td>
<td>1.5</td>
<td>0.4</td>
<td>-</td>
<td>12.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Erucic</td>
<td>22:1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>34.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Most of the analyses gave values similar to those reported in the literature (see Carroll and Khor, 1971 for references). Butter normally contains about 4% of 4:0, and 1-2% each of 6:0 and 8:0, but these short-chain acids were not determined under the conditions used for our analysis. Other minor peaks in butter and tallow were included in the calculations but are not shown in the Table.
significantly more tumors per tumor-bearing rat than the groups on butter and lard diets. However, the group on rapeseed oil diet was exceptional in that it had the lowest number of tumors per rat, and the lowest number of tumors per tumor-bearing rat, and the number of total tumors per rat and the number of tumors per tumor-bearing rat differed significantly from most of groups on diets containing unsaturated fats and oils but not significantly from those on diets containing saturated fats and oils (Table III). The latent periods of the tumors in different dietary groups were variable and there were no significant differences.

Although the results obtained with various groups of rats on semisynthetic diets containing 20% saturated and unsaturated fats are somewhat variable, there is an apparent trend toward higher tumor yields with increasing unsaturation in the dietary fats and oils. This tendency can be easily seen in Fig. 4. It should be noted that rapeseed oil is an obvious exception to this generalization.

Most of the mammary tumors obtained in this study were adenocarcinomas and the higher yield on diets containing unsaturated fats was confined to this type of tumors. Fibroadenomas and adenomas were provoked in small numbers in every dietary group and there was no apparent trend toward higher yield with increasing unsaturation in the dietary fats in these two types of mammary tumors (Table IV).

**Effect of Dietary Fat on Mammary Carcinogenesis induced by Intravenous Injection of DMBA.**

On arrival, 60 female rats were divided into two groups of 30 each. One group was put on high fat diet (20% corn oil) and the group was put on low fat diet (5% corn oil). At 50-52 days of age, each rat was injected, through the tail vein, with a single dose of 5 mg. of
Fig. 4. Effect of different dietary fats and oils fed at the level of 20% by weight of the diet on the rate of appearance of palpable mammary tumors in female rats treated with DMBA.
DMBA in 1 ml. of fat emulsion while the rat was under light ether anaesthesia. The animals were maintained on semisynthetic diets at the time of carcinogen treatment. After treatment with the carcinogen, the animals were returned to their cages immediately and given the same diet which they were fed on before treatment with carcinogen until the end of the experiment. The rats were checked regularly for palpable tumors and the experiment was terminated four months after DMBA administration.

Two rats in each group died within one week after DMBA administration, probably due to the toxic effect of DMBA, but the other 28 rats in each group survived and appeared healthy throughout the experiment. Therefore, all final results were calculated on the basis of 28 rats in each group. The cumulative palpable mammary tumor incidence of rats on high and low corn oil diets is shown in Fig. 5, and the results at autopsy are summarized in Tables VI and VII. The rats grew well on the high and low corn oil diets and there was no significant difference in the mean body weight in the two groups of rats. All rats on high corn oil diet had palpable mammary tumors 3 months after carcinogen treatment, whereas only 82.1% of the rats on low corn oil diet had mammary tumors by this time (Fig. 5). At autopsy, the mammary tumor incidence of the low corn oil group was 89.2% and the difference between high and low corn oil groups was not statistically significant. The rats on high corn oil diet also had more tumors per rats, more tumors per tumor-bearing rats and a shorter latent period than the rats low corn oil diet. The differences was not large enough to be statistically significant. The high corn oil diet appeared to accelerate the occurrence of palpable mammary tumors (Fig. 6). 98% of the tumors found in both high and low corn oil
Fig. 5. Effect of high fat diet (20% corn oil) and low fat diet (5% corn oil) on the cumulative palpable mammary tumor incidence induced in female rats by a single intravenous injection of 5 mg. of DMBA-fat emulsion.
Table VI. Effect of dietary fat on mammary tumor incidence induced in female rats by a single dose of 5 mg. of DMBA injected intravenously.

<table>
<thead>
<tr>
<th>Corn oil diet*</th>
<th>Body Weight (gm.)**</th>
<th>Tumor Incidence†</th>
<th>No. of Tumors per Rat***</th>
<th>No. of Tumors per Tumor-bearing Rat**</th>
<th>Latent Period** (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td>Total</td>
<td>Palpable</td>
<td>Total</td>
</tr>
<tr>
<td>20%</td>
<td>42.3±0.8</td>
<td>240.1±4.0</td>
<td>100.0</td>
<td>100.0</td>
<td>7.0±0.73</td>
</tr>
<tr>
<td>5%</td>
<td>42.9±0.7</td>
<td>238.3±2.2</td>
<td>89.2</td>
<td>89.2</td>
<td>5.9±0.65</td>
</tr>
</tbody>
</table>

* Twenty eight rats in each group.

** Mean ± S.E.M.

† Per cent of rats with mammary tumors.
Table VII. Effect of dietary fat on the incidence of different types of mammary tumors induced by a single dose of 5 mg. of DMBA injected intravenously.

<table>
<thead>
<tr>
<th>Fat Type</th>
<th>Adenocarcinomas Palpable Nonpalpable Total</th>
<th>Adenomas Palpable Nonpalpable Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn oil diet 20%</td>
<td>157 34</td>
<td>191 0</td>
</tr>
<tr>
<td>Corn oil diet 5%</td>
<td>126 39</td>
<td>165 0</td>
</tr>
</tbody>
</table>
Fig. 6. Effect of high fat diet (20% corn oil) and low fat diet (5% corn oil) on the rate of appearance of palpable mammary tumors in female rats induced by a single intravenous injection of 5 mg. of DMBA-fat emulsion.
groups were adenocarcinomas and the difference between the two groups can only be seen in this type of tumor (Table VII).

**Mammary Carcinogenesis in Female Rats Switched to High Corn Oil Diet at Different Intervals after DMBA Administration.**

Four groups of 30 female rats each were fed low fat diet (5% corn oil) after weaning at 20-31 days of age. They were treated at 50 days of age with 5 mg. of DMBA as mentioned in 'Methods'. One day after the administration of DMBA, all 4 groups were returned to low corn oil diet, but 1, 2 and 4 weeks later, group I (△—△), group II (○—○) and group III (●—●) respectively were transferred from low corn oil diet to high fat diet (20% corn oil). Group IV (■—■) was maintained on low corn oil diet throughout the experimental period to serve as control. This experiment was terminated and the tumors were handled as described in 'Methods'.

The cumulative palpable mammary tumor incidence in female rats changed to high corn oil diet at different intervals after DMBA administration is shown in Fig. 7. Transferring female rats from low corn oil diet to high corn oil diet one week after DMBA administration (group I, △—△), resulted in a slight increase in palpable mammary tumor incidence (13%) at autopsy as compared to controls (group IV, ■—■) on low corn oil diet. Group I (△—△) had a significantly higher palpable tumor incidence than group IV (■—■), the control, two months after DMBA administration and this significant difference in palpable tumor incidence between group I (△—△) and group IV (■—■) was maintained as far as three months after DMBA administration. Thereafter the difference became insignificant. At some points between the
Fig. 7. Effect of switching female rats from low fat diet (5% corn oil) to high fat diet (20% corn oil) at different intervals after DMBA administration on the cumulative palpable mammary tumor incidence.

Group I (Δ---Δ)     Group II (o---o)

Group III (Δ---Δ)   Group IV (●---●)
second and the third month after DMBA administration, group I (Δ→Δ) also had a significantly palpable tumor incidence than group II (o→o) and group III (Δ→Δ).

Transferring rats from low corn oil diet to high corn oil diet 2 weeks after DMBA administration (group II, o→o) resulted in an increase in palpable tumor incidence comparable to that of group I (Δ→Δ) which was not significantly different from that of the control, group IV (e→e). At two points after DMBA administration, that is, at the 80th and the 91st day, the difference in palpable tumor incidence between group II (o→o) and group IV (e→e) was significant (P<0.05). However, transferring rats from low corn oil diet to high corn oil diet 4 weeks after DMBA treatment (group III, Δ→Δ) did not produce any difference in palpable tumor incidence at any point during the course of this experiment and the palpable tumor incidence at autopsy was the same as the control (group IV, e→e).

The complete results at autopsy are summarized in Tables VIII and IX. The rats in all four groups grew well and appeared healthy throughout the experiment. They had comparable mean body weight at autopsy and there was no significant difference in mean body weight at any point during the course of this experiment. At autopsy, group I (Δ→Δ), group II (o→o) and group III (Δ→Δ) had total tumor incidence insignificantly higher than group IV (e→e), the control. However, the difference in the number of tumors per rat between group I (Δ→Δ) and group II (o→o) was significant (P<0.05), and a greater significant difference in the number of tumors per rat between group I (Δ→Δ) and group III (Δ→Δ) and between group I (Δ→Δ) and group IV
Table VIII. Effect of changing from low corn oil (5% by weight) diet to high corn oil (20% by weight) diet at different intervals after DMBA administration on mammary tumor incidence in rats.

<table>
<thead>
<tr>
<th>Group*</th>
<th>Body Weight(gm.)**</th>
<th>Tumor Incidence†</th>
<th>No. of Tumors per Rat**</th>
<th>No. of Tumors per Tumor-bearing Rat**</th>
<th>Latent Period** (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Final</td>
<td>Total</td>
<td>Palpable</td>
<td>Total</td>
</tr>
<tr>
<td>I</td>
<td>48.4±0.8</td>
<td>249.2±4.0</td>
<td>96.6</td>
<td>90.0</td>
<td>5.1±0.6</td>
</tr>
<tr>
<td>II</td>
<td>49.4±0.8</td>
<td>250.7±3.1</td>
<td>93.3</td>
<td>86.6</td>
<td>3.6±0.4</td>
</tr>
<tr>
<td>III</td>
<td>49.5±0.6</td>
<td>250.5±9.1</td>
<td>86.6</td>
<td>76.6</td>
<td>3.1±0.4</td>
</tr>
<tr>
<td>IV</td>
<td>48.7±0.6</td>
<td>252.0±2.8</td>
<td>76.6</td>
<td>76.6</td>
<td>2.7±0.4</td>
</tr>
</tbody>
</table>

*Thirty rats in each group. Groups I, II and III were transferred to high corn oil diet 1, 2 and 4 weeks after DMBA administration. Group IV was maintained on low corn oil diet throughout the experiment to serve as control.

**Mean ± S.E.M.

† Per cent of rats with mammary tumors.
Table IX. Effect of changing from low corn oil (5% by weight) diet to high corn oil (20% by weight) diet at different intervals after DMBA administration on the incidence of different types of mammary tumors in rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Adenocarcinomas</th>
<th>Fibroadenomas</th>
<th>Adenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Palpable</td>
<td>Nonpalpable</td>
<td>Total</td>
</tr>
<tr>
<td>I</td>
<td>119</td>
<td>33</td>
<td>152</td>
</tr>
<tr>
<td>II</td>
<td>77</td>
<td>26</td>
<td>103</td>
</tr>
<tr>
<td>III</td>
<td>57</td>
<td>29</td>
<td>86</td>
</tr>
<tr>
<td>IV</td>
<td>50</td>
<td>28</td>
<td>78</td>
</tr>
</tbody>
</table>
were obtained ($P<0.01$). Similarly, the difference in the
to number of tumors per tumor-bearing rat between group I (Δ—Δ) and
group II (o—o) was significant at the 5% level while the differences
in the number of tumors per tumor-bearing rat between group I (Δ—Δ)
and group III (Δ—Δ) and between group I (Δ—Δ) and group IV (o—o)
were significant at the 2.5% level. The difference in tumor latent
period between group I (Δ—Δ) and group IV (o—o) was significant
($P<0.05$), but the differences in tumor latent period between group I
(Δ—Δ) and group III (Δ—Δ) and between group II (o—o) and group IV
(o—o) were not large enough to be statistically significant. It is
interesting, however, that group II (o—o) had more tumors per rats,
more tumors per tumor-bearing rat and a shorter latent period than
group III (Δ—Δ) and group IV (o—o), but the differences were not
statistically significant. Group III (Δ—Δ) and group IV (o—o),
on the other hand, gave comparable results.

Switching the animals to high corn oil diet 1, 2 and 4 weeks
after DMBA administration appeared mainly to accelerate the appearance
of palpable mammary tumors (Fig. 8), since the number of nonpalpable
tumors found at autopsy was not much difference among the four groups.
Irrespective of the type of diets fed to the animals, about 93% of
the mammary tumors induced after DMBA treatment were adenocarcinomas
and it is in this type of tumor that the differences between the groups
were seen (Table IX). Fibroadenomas and adenomas were present in small
numbers in all groups, but no definite trend was seen in these two types
of tumors.
Fig. 8. Effect of switching female rats from low fat diet (5% corn oil) to high fat diet (20% corn oil) at different intervals after DMBA administration on the rate of appearance of palpable mammary tumors.

Group I (Δ--Δ)  Group II (○--○)  
Group III(Δ--Δ)  Group IV (●--●)
Mammary Carcinogenesis in Female Rats switched to Low Corn Oil Diet at Different Intervals after DMBA Administration.

Four groups of 30 weaned female rats each were put on high fat diet (20% corn oil) on the day of arrival. At 50 days of age, the rats were treated with 5 mg. of DMBA as described in 'Methods'. One week after the administration of DMBA, group VI (○○○) was transferred from high corn oil diet to low fat diet (5% corn oil) and thereafter maintained on this diet. Similarly, group VII (●●●) and group VIII (▲▲▲) were switched to low corn oil diet at 2 and 4 weeks after DMBA administration respectively. Group V (▲▲▲) was maintained on high corn oil diet throughout the entire experimental period to serve as control. The experiment was terminated and the tumors were examined as described in 'Methods'.

The cumulative palpable mammary tumor incidence in female rats switched to low corn oil diet at different intervals after DMBA administration is shown in Fig. 9. Group VI (○○○) which was transferred from high corn oil diet to low corn oil diet 1 week after DMBA administration had consistently, although not significantly, lower palpable mammary tumor incidence than the control, group V (▲▲▲), throughout the entire course of this experiment. Group VII (●●●) and group VIII (▲▲▲) also had mammary tumor incidence lower than the control, group V (▲▲▲), but the differences were not large enough to be significant at the 5% level. As a matter of fact, the final palpable tumor incidence at autopsy were the same for all the three groups that were transferred to low corn oil diet.

The complete results at autopsy are summarized in Tables X and
Fig. 9. Effect of switching female rats from high fat diet (20% corn oil) to low fat diet (5% corn oil) at different intervals after DMBA administration on the cumulative palpable mammary tumor incidence.

Group V (Δ---Δ)       Group VI (○---○)
Group VII (●---●)     Group VIII (Δ---Δ)
XI. There was no significant difference in the mean body weight between rats on high and low corn oil diets at any point during the course of this experiment. At autopsy, group V (Δ—Δ), the control group attained 100% total tumor incidence but this was not significantly higher than the total tumor incidence in the other three groups. However, there was an apparent tendency for the total tumor incidence to approach the level in control rats as the transfer from high corn oil diet to low corn oil diet was delayed from one to four weeks after DMBA administration (Table X).

As indicated in Table X, group VI (o—o) had less tumors per rat, less tumors per tumor-bearing rat and a longer latent period than the control, group V (Δ—Δ), but the differences were not significant at the 5% level. Similarly group VII (e—o) and group VIII (Δ—Δ) also had less tumors per rat, less tumors per tumor-bearing rat and a longer latent period than the control, but almost all the differences were not significant except the difference in the number of palpable tumors per rat between group V (Δ—Δ) and group VIII (Δ—Δ).

According to our hypothesis that high corn oil diet increases and low corn oil diet lowers the tumor yield, one would expect a smaller value in all the parameters mentioned above for group VII (e—e) and group VIII (Δ—Δ), compared to group VI (o—o). However, this is apparently not the case since the number of tumors per rat and the tumors per tumor-bearing rat in both group VII (e—e) and group VIII (Δ—Δ) were slightly smaller than those of group VI (o—o) (Table X).

The effect of low corn oil diet fed to animals beginning at different intervals after DMBA administration appeared mainly in
Table X. Effect of switching from high corn oil (20% by weight) diet to low corn oil (5% by weight) diet at different intervals after DMBA administration on mammary tumor incidence in rats.

<table>
<thead>
<tr>
<th>Group*</th>
<th>Body Weight (gm.)**</th>
<th>Tumor Incidence† Total Palpable</th>
<th>No. of Tumors per Rat** Total Palpable</th>
<th>No. of Tumors per Tumor-bearing Rat**</th>
<th>Latent Period** (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>39.7±0.6</td>
<td>245.7±2.7</td>
<td>100.0</td>
<td>100.0</td>
<td>5.4±0.6</td>
</tr>
<tr>
<td>VI</td>
<td>39.8±0.6</td>
<td>236.2±4.0</td>
<td>86.6</td>
<td>86.6</td>
<td>4.2±0.5</td>
</tr>
<tr>
<td>VII</td>
<td>40.6±0.7</td>
<td>240.2±3.4</td>
<td>93.3</td>
<td>86.6</td>
<td>4.0±0.6</td>
</tr>
<tr>
<td>VIII</td>
<td>40.1±0.6</td>
<td>237.1±2.9</td>
<td>96.6</td>
<td>86.6</td>
<td>4.0±0.5</td>
</tr>
</tbody>
</table>

*Thirty rats in each group. Group V was maintained on high corn oil diet throughout the experiment.

Groups VI, VII and VIII were transferred to low corn oil diet 1, 2 and 4 weeks after DMBA administration.

**Mean ± S.E.M.

†Percent of rats with mammary tumors.
Table XI. Effect of switching from high corn oil (20% by weight) diet to low corn oil (5% by weight) diet at different intervals after DMBA administration on the incidence of different types of mammary tumors in rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Adenocarcinomas</th>
<th>Fibroadenomas</th>
<th>Adenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Palpable</td>
<td>Nonpalpable</td>
<td>Total</td>
</tr>
<tr>
<td>V</td>
<td>122</td>
<td>31</td>
<td>153</td>
</tr>
<tr>
<td>VI</td>
<td>89</td>
<td>30</td>
<td>119</td>
</tr>
<tr>
<td>VII</td>
<td>88</td>
<td>30</td>
<td>118</td>
</tr>
<tr>
<td>VIII</td>
<td>76</td>
<td>32</td>
<td>108</td>
</tr>
</tbody>
</table>
decreasing the yield of palpable mammary tumors (Fig. 10) since there was not much difference in the number of nonpalpable mammary tumors found at autopsy among the four groups (Table X). In this experiment, over 90% of the mammary tumors occurring in each group were adeno-carcinomas. Fibroadenomas and adenomas occurred in small numbers in each group (Table XI).

**Mammary Carcinogenesis in Female Rats Switched to Low Corn Oil Diet Two Months after DMBA Administration.**

Two groups of 30 female rats were placed on high fat diet (20% corn oil) after weaning at 21-22 days of age. They were treated with 5 mg. of DMBA at 50 days of age and were maintained on the high corn oil diet after carcinogen treatment. Two months later, one group H-L, was switched to low fat diet (5% corn oil) and this was continued until the end of the experiment. The other group, H-H, was kept on high corn oil diet throughout the experiment to serve as control. The experiment was terminated four months after DMBA administration.

The results at autopsy are summarized in Table XII. Transferring rats from high corn oil diet to low corn oil diet two months after DMBA administration did not produce much difference in the incidence of mammary tumors between the experimental group and the control. During the course of this experiment, it was also observed that low corn oil diet did not seem to affect the growth of well-established mammary tumors that developed before the change of diet. However, it seemed to increase slightly the number of nonpalpable tumors although the total tumor yield was not much different.
Fig. 10. Effect of switching female rats from high fat diet (20% corn oil) to low fat diet (5% corn oil) at different intervals after DMBA administration on the rate of appearance of palpable mammary tumors.

Group V (Δ---Δ)    Group VI (o---o)
Group VII (●---●)  Group VIII (Δ---Δ)
Table XII. Effect of switching rats from high corn oil (20% by weight) diet to low corn oil (5% by weight) diet two months after DMBA administration on mammary tumor incidence.

<table>
<thead>
<tr>
<th>Group</th>
<th>Palpable Tumor Incidence before change of diet</th>
<th>Palpable Tumor Incidence after change of diet</th>
<th>Total Tumor Incidence at autopsy †</th>
<th>No. of Palpable Tumors appeared before change of diet</th>
<th>No. of Palpable Tumors appeared after change of diet</th>
<th>No. of Nonpalpable Tumors found at autopsy</th>
<th>Total No. of Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>H−H</td>
<td>48.2</td>
<td>38.0</td>
<td>89.6</td>
<td>27</td>
<td>67</td>
<td>17</td>
<td>111</td>
</tr>
<tr>
<td>H−L</td>
<td>48.2</td>
<td>27.6</td>
<td>82.7</td>
<td>21</td>
<td>54</td>
<td>42</td>
<td>117</td>
</tr>
</tbody>
</table>

*Twenty nine rats in each group. H−H was fed high corn oil diet throughout the experiment and H−L was transferred from high corn oil diet to low corn oil diet two months after DMBA administration.

†Per cent of rats with mammary tumors.
The Distribution of Radioactivity in Tissues of 50-60 day old Female Rats after Injection of Tritiated Estradiol-17β.

a. Intramuscular Injection.

Female S-D rats were fed high fat diet (20% corn oil) and low fat diet (5% corn oil) beginning at 21-22 days of age. At 50-60 days of age, vaginal smear was done daily and rats at diestrus cycle were injected with a single dose of 1 million counts of estradiol-17β-6,7-3H (0.1 μg.) in 0.5 ml. of 5% ethanolic saline into the thigh muscle of the hind limb between 1.30 p.m. and 3.00 p.m. At intervals of 1/2, 1 and 2 hours after injection, the radioactivity in several tissues was analyzed as described in 'Methods'. The results are shown in Fig. 11.

In all tissues examined, the radioactivity was consistently higher in the low corn oil group than in the high corn oil group. The differences were greater in the pituitary and uterus, but unfortunately the differences were not statistically significant at the 5% level except in the uterus at 1 hour after injection. (P<0.05)

b. Intravenous Injection.

After completion of the above experiment, the question arose whether the observed differences in the distribution of radioactivity in tissues of rats fed on high and low corn oil diets might be due to differences in the rate of diffusion of radioactive estradiol into the circulation from the site of injection. Thus, another experiment was performed in which the same amount of tritiated estradiol was injected through the tail vein while the rat was under light ether anaesthesia. The results obtained at 1/2 and 1 hour after injection are shown in Fig. 12.
Fig. 11. Effect of high fat diet (20% corn oil) and low fat diet (5% corn oil) on the distribution of radioactivity in various tissues of female rats after intramuscular injection of tritiated estradiol-17β. (50-60 day old rats)

Note: The value shown at each interval in each group is the 'Mean' of five rats ± S. E. M.
Distribution of $^3$H Estradiol after Intramuscular Injection
(50-60 day old female rats)

- 20% Corn oil diet
- 5% Corn oil diet

- Mammary Glands
- Liver
- Blood
- Uterus

- Pituitary
- Ovaries
- Adrenals

Time in hours: 1/2, 1, 2

cpm x 100/100 mg wet weight
Fig. 12. Effect of high fat diet (20% corn oil) and low fat diet (5% corn oil) on the distribution of radioactivity in various tissues of female rats after intravenous injection of tritiated estradiol-17β. (50–60 day old rats)

Note: The value shown at each interval in each group is the 'Mean' of five rats ± S. E. M.
Distribution of $^3$H Estradiol after Intravenous Injection.
(50-60 day old female rats)

- 20% corn oil diet
- 5% corn oil diet

- Blood
- Mammary glands
- Pituitary
- Ovaries
- Adrenals
- Liver
- Uterus

Time (hour)

$\text{cpm} \times 10^3 / \text{mg wet weight}$
The results were in general similar to those obtained in the previous experiment. The radioactivity tended to be higher in the low corn oil group than in the high corn oil group. The differences, however, were not significant statistically. The levels of radioactivity in the various tissues, irrespective of the kinds of diet the rats consumed, were somewhat higher in the present experiment compared to those obtained in the previous experiment when the same amounts of radioactivity was injected intramuscularly.

Results obtained in the above two experiments, therefore, suggested that there was a difference in the distribution of radioactive estradiol in the tissues of rats fed on high and low corn oil diets and this difference was not affected, to any great extent, by the route of administration of the labelled estradiol.

The Distribution of Radioactivity in Tissues of 90-100 Day Old Female Rats after Intravenous Injection of Tritiated Estradiol-17β.

Female S-D rats were fed high fat diet (20% corn oil) and low fat diet (5% corn oil) beginning at 50-51 days of age. At 90-100 days of age, each rat was injected with a single dose of 2 million counts of tritiated estradiol-17β (0.2 μg) in 1 ml. of 5% ethanolic saline through the tail vein while the rat was under light ether anaesthesia. The estrus cycle of the rats was checked daily between 90-100 days by vaginal smear and the experiment was carried out with rats at diestrus cycle only. At intervals of 1/2, 1 and 2 hours after injection, the radioactivity present in the tissues was analyzed as described in 'Methods'. The results are shown graphically in Fig. 13.

The results are somewhat variable. In the adrenals, mammary
Fig. 13. Effect of high fat diet (20% corn oil) and low fat diet (5% corn oil) on the distribution of radioactivity in various tissues of female rats after intravenous injection of tritiated estradiol-17β. (90-100 day old rats)

Note: The value shown at each interval in each group is the 'Mean' of five rats ± S. E. M.
Distribution of $^3H$ Estradiol after Intravenous Injection.
(90-100 day old female rats)
gland and the uterus, the radioactivities were somewhat higher in the low corn oil group than the high corn oil group. However, in the ovaries, pituitary and liver, the radioactivities were slightly lower in the low corn oil group than in the high corn oil group. Most of the differences were not statistically significant except the difference of radioactivity in the uterus at 1 hour after injection. (P<0.05).

Results obtained in the present experiment, in comparison to those obtained previously with 50-60 day old rats, suggested that there was a greater variability in the hormonal status in older rats. However, it is interesting to note that the differences in the uptake of radioactivity in the uterus in rats fed on high and low corn oil diets are maintained in the two age groups.

The Distribution of Radioactivity in Tissues of 90-100 Day Old Female Rats after Intravenous Injection of Tritiated Progesterone.

Ninety to one hundred day old female rats which had been fed on high fat diet (20% corn oil) and low fat diet (5% corn oil) since 50 days of age were used in this study. Rats in estrus were injected with a single dose of 2 million counts of progesterone-1,2-3H (0.19 µg) in 1 ml. of 5% ethanolic saline through the tail vein while the rats were under light ether anaesthesia between 1.30 p.m. and 3.00 p.m. At 1/2, 1 and 2 hours after injection, the rats were sacrificed by decapitation. The tissues were collected and analyzed as described in 'Methods'. The results of this study are shown graphically in Fig. 14.

The overall radioactivity in the tissues was relatively low except in the liver, compared with the radioactivity in similar tissues when the same amounts of counts of tritiated estradiol-17β were in-
Fig. 14. Effect of high fat diet (20% corn oil) and low fat diet (5% corn oil) on the distribution of radioactivity in various tissues of female rats after intravenous injection of tritiated progesterone. (90-100 day old rats)

Note: The value shown at each interval in each group is the average of three rats.
Distribution of $^3$H Progesterone after Intravenous Injection.
( 90-100 day old female rats )

![Graph showing the distribution of $^3$H Progesterone over time in different organs and with different diets.](image-url)
jected. There was no selective uptake of radioactivity in the pituitary and the uterus as noted in the case of the distribution of tritiated estradiol. No consistent difference in the accumulation of radioactivity in various tissues between the two dietary groups could be seen. Although at 1 hour after injection, the mammary gland of rats fed on high corn oil diet appeared to accumulate more radioactivity than the mammary gland of rats fed on low corn oil diet, the difference was not significant. In the liver, the low corn oil group appeared to have higher radioactivity than the high corn oil group, but again the difference was not significant.

**Metabolism of Estradiol-4-\(^{14}\)C in the Liver of Female Rats in Vivo**

In view of the finding that several tissues of rats on low corn oil diet accumulated more radioactivity than similar tissues in rats on high corn oil diet after injection of labelled estradiol-\(^{17}\beta\), a preliminary experiment was carried out to see if there was any difference in the metabolism of labelled estradiol in the liver. Rats, between 90 and 100 days of age, which had been fed either on high corn oil diet or low corn oil diet were injected with 1 million counts of estradiol-4-\(^{14}\)C intravenously and the radioactivity in the liver 1 hour after injection was analyzed as described in 'Methods'. The results are shown in Table XIII.

The liver of rats fed on high corn oil diet converted more of the labelled estradiol to its water soluble metabolites than the liver of rats fed on low corn oil diet 1 hour after injection. The difference was highly significant. (P<0.001) The chloroform fraction
Table XIII. Metabolism of estradiol-4-\(^{14}\)C in the liver of female rats fed on high and low corn oil diets.

<table>
<thead>
<tr>
<th>Corn Oil Diet*</th>
<th>Radioactivity in Liver** (cpm per 100 mg. wet weight)</th>
<th>Per Cent of Total Radioactivity (cpm) in Liver Remaining in Chloroform Fraction**</th>
<th>Per Cent of Total Radioactivity (cpm) in Liver Remaining in Methanol-water Fraction **</th>
</tr>
</thead>
<tbody>
<tr>
<td>20%</td>
<td>251.2 ± 46.3</td>
<td>43.1 ± 0.7</td>
<td>56.3 ± 1.4</td>
</tr>
<tr>
<td>5%</td>
<td>236.1 ± 42.3</td>
<td>52.4 ± 0.8</td>
<td>46.3 ± 1.2</td>
</tr>
</tbody>
</table>

*Five rats in each group.

**Mean ± S.E.M.
presumably contained the free estrogens while the methanol-water fraction contained the estrogen conjugates and its polar metabolites. No attempt was made to characterize the components of the two fractions at this moment. Since it had been found by other investigators that the intact labelled estradiol accounted for more than 90% of the radioactivity retained in the mammary gland, pituitary and uterus, the difference in the conversion of labelled estradiol to its water soluble metabolites in the liver of rats on high and low corn oil diets, therefore, appeared to account for the differences in accumulation of radioactivity in tissues of the two dietary groups.

**Effect of Dietary Fat on the Level of Plasma Lipids in Female Rats**

Blood was drawn from 90-100 day old female rats which had been fed on either high fat diet (20% corn oil) or low fat diet (5% corn oil) beginning at 50 days of age. Plasma was prepared and plasma lipids were analyzed as described in 'Methods'.

It should be noted here that two lots of rats were used in this study. The first lot consisted of 10 female rats which were divided into 2 groups of 5 each and placed on high and low corn oil diets. These rats were used in the determination of total lipids, phospholipids, neutral lipids, cholesterol and triglycerides in plasma. The other lot consisted of 14 rats which were divided into 2 groups of 7 each and placed on high and low corn oil diets. These rats were used for the determination of plasma free fatty acids. The results are shown in Table XIV.

There was not much difference in total lipids, phospholipids,
Table XIV. Effect of dietary corn oil on the level of plasma lipids in female rats.

<table>
<thead>
<tr>
<th>Corn Oil Diet</th>
<th>Total Lipids* (mg./100 ml. plasma)</th>
<th>Phospholipids* (% of total lipids)</th>
<th>Neutral Lipids* (% of total lipids)</th>
<th>Cholesterol* (mg./100 ml. plasma)</th>
<th>Triglycerides* (mg./100 ml. plasma)</th>
<th>Plasma Free Fatty Acids** (µ Eq./litre plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20%</td>
<td>300±11.7</td>
<td>51.8±1.5</td>
<td>46.8±2.0</td>
<td>6.4±1.0</td>
<td>60.5±4.8</td>
<td>52.4±5.9</td>
</tr>
<tr>
<td>5%</td>
<td>338±14.2</td>
<td>51.5±2.4</td>
<td>48.2±2.6</td>
<td>7.1±1.7</td>
<td>65.9±8.1</td>
<td>86.0±10.3</td>
</tr>
</tbody>
</table>

* Five rats in each group. Mean ± S.E.M.

** Seven rats in each group. Mean ± S.E.M.
neutral lipids and cholesterol level in plasma of rats fed on high and low corn oil diets. However, rats on low corn oil diet had a significantly (P<0.05) higher plasma triglyceride level and a significantly lower plasma free fatty acid level than rats on high corn oil diet.
DISCUSSION

Mammary tumors can be readily induced in female S-D rats by a single intragastric administration of DMBA (Huggins et al., 1961a). These tumors have structures resembling human breast cancers (Young et al., 1963; Daniel and Prichard, 1964a) and they respond to hormonal therapy in a way similar to that of human breast cancer (Huggins, 1967). In addition to the above features, Huggins' method of mammary tumor induction is simple and easy to apply. Since only a single administration of the carcinogen is required to provoke neoplastic transformation in the mammary tissue, this model provides an excellent opportunity for studying the stages of carcinogenesis separately. Therefore, we have used this experimental model in our studies on the relationship of dietary fat and mammary carcinogenesis in female rats.

Mammary carcinogenesis in the rat has been found to be affected by a number of factors (Noble and Cutts, 1959; Dao, 1964a, 1969a). The factor of dietary fat has been observed since the early 1940, but it has not been pursued very far in the following years. As a result, the mechanism of action of dietary fat on mammary carcinogenesis remains largely unknown. Results obtained previously in our laboratory (Gammal et al., 1967; Carroll and Khor, 1970) and those obtained in the present studies have added new information to the understanding of this problem.

The results shown in Figs. 1 and 2 and Tables I and II confirm our previous findings (Gammal et al., 1967; Carroll and Khor, 1970) that young adult female S-D rats treated with DMBA have higher mammary tumor inciden
ce, shorter latent period and more palpable mammary tumors on a semisyn-
thetic diet containing 20% by weight of corn oil than a comparable diet
containing 0.5% corn oil and provide additional information on the effects
of intermediate levels of dietary corn oil. Raising the level of corn oil
from 0.5% to 5% did not increase the tumor incidence (i.e. percentage of
rats with mammary tumors), and the number of palpable tumors appreciably,
but a further increase to 10% increased the tumor incidence and the number
of mammary tumors markedly. Actually the rats on 10% corn oil diet had
slightly higher mammary tumor incidence and more palpable mammary tumors
than those on 20% corn oil diet (Figs. 1 and 2). These findings are in
general agreement with the results of Silverstone and Tannenbaum (1950)
who investigated the effect of diets containing different levels of
partially hydrogenated cottonseed-soybean oil on the incidence of spont-
aneous mammary tumors in mice. They found that the tumor incidence in-
creased as the level of fat in the diet was raised from 2-4% up to 12-16
%, but beyond this level additional fat seemed to have little effect.
Both in our experiments and those of Silverstone and Tannenbaum, about
90% of the animals developed mammary tumors when the diet contained 10-
12% fat and it might be argued that there is not much room for further
increase in tumor incidence at higher level of dietary fat. A smaller
dose of DMBA could have been used in our studies to give a lower inciden-
ce, but the 5 mg. dose was chosen because it appeared to give the best
differential in tumor yield between rats on high and low corn oil diets
within the chosen experimental period (Carroll and Khor, 1970). It
may also be noted that the number of mammary tumors per rat showed a
significant increase as the level of dietary corn oil was raised
from 5% to 10%, but no further increase in going from 10% to
20% (Table II). The effect of raising the level of corn oil in the diets was reflected mainly in the number of adenocarcinomas; fibroadenomas and adenomas were both unaffected (Table II).

The studies of Gammal et al (1967) in relation to dietary fat and the incidence of mammary tumors in rats indicated that coconut oil had much less effect on mammary carcinogenesis than corn oil. Other workers had shown that edible oil such as olive oil (Benson et al, 1956), Crisco (Dunning et al, 1949) and lard (Engel and Copeland, 1951) all enhanced mammary carcinogenesis in the rat, but no systematic studies have been carried out with different dietary fats. Our investigation was therefore expanded to include a variety of other edible fats and oils. The results indicate that, in general, rats on diets containing unsaturated fats developed more mammary tumors after treatment with DMBA than rats on similar diet containing saturated fats (Fig. 4. Table III). In fact, the tumor yield with dietary fat such as coconut oil and tallow were much the same as those obtained with low corn oil diet (Fig. 2 and 4). Although the total tumor yield was lower with saturated fats, in most cases the percentage of animals developing mammary tumors was about the same as for unsaturated fats (Fig. 3). The main differences were therefore mainly in the number of tumors per rats (Table III).

Rapeseed oil was exceptional in giving a low tumor yield although it contains a relatively high proportion of unsaturated fatty acids. Much of unsaturation, however, is accounted for by the C₂₀ and C₂₂ monoens, eicosenoic acid and erucic acid (Table V). Rapeseed oil had been reported to have a lower digestability than most other dietary fats and oils (Deuel, 1955) and was shown to depress growth in rats
(Thomasson and Boldingh, 1955) and to increase the concentration of cholesterol in the adrenals and ovaries (Carroll and Noble, 1952). Possibly the low tumor yield is related to some of these effects.

In the present experiments (Table III), the animals in rapeseed oil group had the lowest average body weight at autopsy although it was not much below that of the other dietary groups. The apparent digestibility was also lower than any other fat except tallow (Table III), but not as low as the value reported in the literature (Deuel et al, 1948). Erucic acid appears to be responsible for most of the observed effects of rapeseed oil in animals (Thomasson et al, 1955), and the relatively small effect on growth and digestibility in the present studies may be due to the use of an oil containing a lower percentage of erucic acid (Downey et al, 1969) or to feeding the oil at a lower level than those used in earlier studies.

Lard appeared to be more effective than other solid fats tested and olive oil, which contains oleic acid as the major unsaturated fatty acid (Table V), gave a tumor yield comparable to that of oils with higher degree of unsaturation (Fig. 4). Experiments are now in progress to study mammary carcinogenesis in rats fed on diets containing rapeseed oil of high and low erucic acid contents and to repeat the experiment using olive oil as dietary fat. Results obtained so far indicate that rats fed on diet containing rapeseed oil of low erucic acid content had higher tumor incidence and more mammary tumors than those fed on diet containing rapeseed oil of high erucic acid content. The tumor incidence and the number of mammary tumors obtained so far in the group fed on 20% olive oil were comparable to those on diet containing rape-
seed oil of high erucic acid content and to those on low fat diet.

We have found in our present studies that both the quantity and quality of fats contribute to the promoting activity of dietary fat. If the degree of unsaturation of fat is a factor in causing the differences in tumor yields between rats on saturated and unsaturated fats and oils, it is logical that a greater differential should be obtained if the fat content of the diets was lowered to, for instance, 10%. Experiments of this sort are definitely needed to provide further information regarding the mechanism of action of dietary fat on mammary carcinogenesis.

Previous studies (Gammal et al, 1967; Carroll and Khor, 1970) performed in our laboratory in relation to dietary fat and mammary carcinogenesis in rats have used mammary tumors induced in female S-D rats by a single intragastric instillation of DMBA. Intravenous injection of DMBA was shown by Huggins et al (1961b) to induce mammary tumors as effectively as intragastric administration of the same carcinogen. We used this technique of mammary tumor induction in one of our studies (Fig. 5 and 6, Tables VI and VII). The rats on high corn oil diet had higher tumor incidence, higher tumor yield and shorter latent period than those on low corn oil diet. However, the differential between the high corn oil group and the low corn oil group was not as great as that usually obtained when the carcinogen was administered intragastrically (Carroll and Khor, 1970). This could be due to the fact that the dose of DMBA used in this experiment was too large since two animals in each group died within one week after administration of DMBA, apparently due to the toxic effect of DMBA; and since the tumor incidence in the high
corn oil group reached 100% three months after DMBB administration (Fig. 5).

On the whole, the results of this study suggest that the tumor-promoting property of high corn oil diet is not dependent on the route of administration of the carcinogen. This is in agreement with other reports in the literature which showed that the tumor-promoting property of high fat diet could be demonstrated with mammary tumors induced by implantation of diethylstilbestrol pellets (Dunning et al, 1949) and by chronic feeding of AAF in the diet (Sugai et al, 1962).

There is much evidence to indicate that mammary carcinogenesis can be divided into stages (Dao, 1962b, 1964a, 1969a). The initiation process of neoplastic transformation appears to be rapid and short. Dao et al (1968) transplanted mammary glands 10 minutes after an intravenous injection of 5 mg. of DMBB and observed development of mammary tumors in 3 out of 8 surviving grafts.

Carroll and Khor (1970) found that feeding high corn oil diet to female rats, beginning one day after the administration of DMBB, enhanced mammary carcinogenesis as much as feeding the same diet throughout the entire experimental period. Similarly low corn oil diet when fed, beginning only one day after the administration of DMBB, lowered the tumor yields to the same extent as the same diet fed throughout the experimental period. Garmsal et al (1968), who measured the uptake of DMBB in mammary tissue after a single intragastric instillation of the carcinogen, found no significant difference in the level of DMBB in rats fed on high and low corn oil diets. Small amounts of DMBB were still detectable in rats on either diets at the fifth day after
administration. However, the level of DMBA in mammary tissue does not represent the actual level of DMBA in the mammary epithelial cells where mammary tumors eventually arise (Janss and Moon, 1970) and only a very small amount of DMBA is apparently required for the induction of mammary tumors (Dao, 1970). Therefore, there is a possibility that high corn oil diet when given to rats beginning one day after DMBA administration might affect the level of DMBA in the mammary epithelial cells. To be sure of our previous conclusion (Carroll and Khor, 1970) that high corn oil diet exerted its effects mainly on the developmental stage of the carcinogenic process, further experiments were carried out in which the change of diets was performed at different intervals after the treatment with carcinogen.

It was found that transferring female rats from low corn oil diet to high corn oil diet beginning one week after DMBA administration resulted in the production of mammary tumors significantly more than those obtained in control rats fed on low corn oil diet (Fig. 7, Table VIII). The tumor yield obtained in this group was comparable to that obtained in previous experiment when rats were transferred from low corn oil diet to high corn oil diet beginning one day after the administration of DMBA (Carroll and Khor, 1970). Delaying the feeding of high corn oil diet until two weeks after DMBA treatment decreased the tumor yield to some extent, but it was still considerable higher (P<0.10) than that of the controls. High corn oil diet when fed to rats beginning later than two weeks after DMBA treatment produced no effect on tumor yield compared to low corn oil control (Fig. 7, Table VIII).

In a converse experiment, it was found that low corn oil diet
lowered considerably the incidence and yield of mammary tumors whether it was fed to rats beginning one, two or four weeks after carcinogen treatment (Fig. 9, Table X). However, it appeared that low corn oil diet which was fed to rats beginning one week after DMBA treatment had greater inhibitory effect on tumor development than when it was fed beginning two and four weeks after DMBA treatment.

Results obtained in the above experiments provide further evidence for our previous conclusion that high corn oil diet exerted its effects mainly on the development of mammary tumors. A similar conclusion was reached by Tannenbaum (1944b) who studied the effect of high fat diet on skin carcinogenesis induced in mice by BP. It is interesting to note that dietary fat appears to have similar promoting effect on the carcinogenesis of two different tissues, the skin and the mammary gland.

It should be noted here that, in the above two experiments, the change of diets was performed before the appearance of any palpable mammary tumor. In a later experiment, in which female rats were transferred from high corn oil diet to low corn oil diet two months after the treatment of DMBA, it was found that low corn oil diet did not produce much difference in the incidence and yield of mammary tumors between the control and the experimental groups. It was observed during the course of this experiment that low corn oil diet did not seem to have any effect on the growth of well-established mammary tumors. However, it seemed to increase slightly the number of nonpalpable tumors although the total tumor yield was not much different. Considering the above results (Table XII) and those found earlier (Tables VIII and X),
it appears that the effects of high corn oil diet on the development of mammary tumors is restricted to the period immediately after the initiation to the stage when the tumors become palpable.

Hyperplastic alveolar nodules (HANs) have been observed in mice since the early 1940's (Gardner, 1942; Huseby and Bittner, 1946). The preneoplastic nature of HANs was first demonstrated by DeOME, Faulkin, Bern and Blair (1959) who transplanted such lesions into gland-free mammary fat pads and observed the development of mammary tumors. HANs were also observed by Dao, Tanaka and Gawlak (1964) in the mammary glands of DMBA-treated rats. However, Beuving, Faulkin, DeOME and Bergs (1967) reported that HANs appeared with greater frequency in female rats treated with DMBA whereas no similar lesions were observed in non-treated controls. The above result was later confirmed by Dao (1969a). Transplanting DMBA-induced HANs into gland-free mammary fat pads, Beuving (1968) and Dao (1969a) both observed that some of these lesions developed into mammary tumors while others remained hyperplastic. All these results support the hypothesis that hyperplastic alveolar nodules (HANs) are the precancerous stage of carcinogen-treated mammary tissues in female rats.

The findings of Beuving (1968) and of Dao (1969a) indicate that the prevailing conditions in the host apparently play a decisive role in the final neoplastic transformation of HANs. Beuving (1969) showed that ovarian hormones were necessary for the formation of HANs, but were not necessary for the maintenance and survival of these lesions in rats. Therefore, it will be of some interest to find out the effects of high and low fat diets on the formation of HANs after the administration of DMBA. Such an approach will definitely shorten the experimental period
and reduce the cost of the experiments since, with the present experimental model, it requires about five months to complete one long-term feeding experiment and hundreds of dollars for the general care and feeding of the animals. Hopefully, this new approach may provide approximately the same kind of information that we are looking for.

Examination of statistical data for humans of different countries revealed a strong positive correlation between dietary fat intake and age-adjusted death from breast cancer (Lea and Birm, 1966; Carroll et al., 1968; Wynder, 1969; Hems, 1970). The aetiology of human breast cancer remains unknown, but factors such as virus, radiation, hormones and chemicals have been suggested by various investigators (De Waard, 1969; Boot, 1970). Mammary cancer induced in female rats by hormones and by chemicals has been shown to respond to alteration of fat content in the diet (Dunning et al., 1949; Gammal et al., 1967; Carroll and Khor, 1970), but there has been no study on the effect of dietary fat on virus-induced and radiation-induced mammary cancer in laboratory animals. Since dietary fat appears to affect mainly the development rather than the initiation of mammary tumors, virus-induced and radiation-induced mammary tumors should also respond to dietary manipulations in the same manner as mammary tumors induced by hormonal and chemical agents. As a matter of fact, skin cancer induced by ultraviolet light irradiation has been shown to be enhanced by feeding a ration high in fat content (Baumann and Rusch, 1939). Therefore, it seems that the promoting effect of high fat diet is also independent of the nature of the carcinogens. If the findings in experimental animals were applicable in humans, they suggest the possibility that the occurrence of breast cancer might be delayed and the incidence decreased by reducing the dietary fat intake. Human breast cancer is characterized by a long latent period and any measure
that can delay the occurrence of the tumor is therefore of practical importance. The high fat diets used in our studies contain about the same level of fat (20% by weight = approximately 40% of total calories) as typical American diets, whereas a low fat diet which was effective in decreasing the percentage of rats with tumors and the number of total tumors is comparable in fat content (5% by weight = 10% by calories) to diets in countries such as Japan where the death from breast cancer is much lower than in America. The fact that unsaturated fats appeared to enhance the yield of mammary tumors to a greater extent than saturated fats in the experiments with rats also suggests that caution should be exercised in recommending a large scale shift to more highly unsaturated fats in human diets in attempt to reduce the risk of atherosclerosis. As a matter of fact, Pearce and Dayton (1971) have shown in an eight-year clinical trial that a diet high in polyunsaturated fats and low in saturated fat and cholesterol increased the incidence of carcinomas in male subjects as compared to human males on control diets, which differed from the experimental diets only in the nature of fat used.

Lavik and Baumann (1941) studied the effect of diet containing either 15% coconut oil or hydrogenated vegetable oil (Primex) on skin carcinogenesis induced in mice by MC and found that the tumor-promoting activity of dietary fat resided mainly in the fatty acid fraction; the glycerol and the unsaponifiable fractions had little or no activity at all. In their study on the effect of dietary fat on AAF-induced carcinogenesis, Sugai et al (1962) noticed that the non-urea adduct-forming fraction or the lipase undigestible fraction of heated corn oil had the greatest tumor-promoting activity. These two fractions were
shown to have polymeric materials and free fatty acids.

Based on the observation that, after DMBA treatment, rats on 20% corn oil diet had significantly higher mammary tumor incidence and tumor yield than rats fed on 20% coconut oil diet, Gammal et al (1967) suggested that the nature of dietary fat might be an important factor in the promotion of mammary carcinogenesis. The results obtained in our present study on the effects of different fats and oils fed at the level of 20% by weight of the diet (Fig. 4, Table III) tended to support the above suggestion. Harman (1969), in a short communication, reported that rats fed on high corn oil diet supplemented with $\alpha$-tocopherol acetate at the level of 20 mg. per 100 gm. of diet had significantly lower tumor incidence than those on a similar diet which was supplemented with $\alpha$-tocopherol acetate at the level of 5 mg. per 100 gm. of diet. From these results, he suggested that lipid peroxidation was involved in the promotion of mammary carcinogenesis by high corn oil diet. In our experiments, the diets were supplemented with $\alpha$-tocopherol acetate at the level of 11 mg. per 100 gm. of diet. The level of vitamin E in our diets is therefore between the levels of vitamin E used by Harman (1969), so it is debatable whether or not lipid peroxidation is involved in the promotion of mammary carcinogenesis by diets containing high level of unsaturated fats. Besides, lipid peroxidation is not the only role of vitamin E under physiological conditions; vitamin E has other functions in vivo (Green and Bunyan, 1969). Therefore, further experiments are required to establish the role of vitamin E in the promotion of mammary carcinogenesis by high fat diets.
The products of lipid peroxidation have long been suspected to be involved in carcinogenesis in laboratory animals and in man. Extensive studies on the carcinogenic property of heated and oxidized fats had been carried out in the past (Artman, 1969; O'gara, 1969), but the cocarcinogenic property of these fats has received relatively little attention. Lavik and Bauman (1941) reported that heat-treated hydrogenated vegetable oil (Primex) added to the diet at the level of 10% by weight of the diet increased markedly the incidence of skin tumors induced by MC in mice from 31% in the group fed fresh untreated oil to 71% in the group fed heat-treated oil. The peroxide value, however, was lower in the heat-treated oil than in the fresh oil. A similar result was obtained by Sugai et al (1962) who found that heat-treated corn oil fed at the level of 10% by weight of the diet considerably enhanced the mammary carcinogenesis induced in rats by AAF, compared to fresh corn oil fed at the same level. The peroxide value was higher in the heat-treated corn oil than the fresh, untreated corn oil. It appears that heat-treated or oxidized fats have greater tumor-promoting activity than fresh, untreated fats.

Dunning et al (1949) and Benson et al (1956) noticed that in histological section rat mammary gland appeared more active in animals on high fat diet compared to those on low fat diet. Tannenbaum (1942b) suggested that dietary fat might have a dual action, namely, solvent action and co-carcinogenic action. For solvent action, he suggested that high fat diet might increase the fat content of the tissue and thus prolong the storage and intensify the exposure of the mammary epithelial cells to the carcinogen. Gammal et al (1968) measured the level of DMBA
in mammary tissue of rats on high and low corn oil diets at different intervals after intragastric instillation of the carcinogen and found not much different in the accumulation and clearance of the carcinogen from the mammary tissue between the two dietary groups. Our present findings (Figs. 7 and 8. Table VIII) showed that high corn oil diet was still very effective in promoting mammary carcinogenesis even if it was fed beginning two weeks after DMBA administration. The above findings, therefore, do not support the solvent action of dietary fat as a factor contributing to the promotional effect of high corn oil diet.

The co-carcinogenic action of dietary fat was not precisely defined by Tannenbaum (1942b). However, he suggested metabolic stimulation of potential tumor cells as a possible action. How dietary fat stimulates potential tumor cells to develop into visible tumors is not known. A diet high in fat content has been shown to affect the lipid composition (Gammal et al., 1967; Khor, 1968) and fatty acid synthesis (Coniglio and Bridges, 1966) of the mammary tissue of female rats. The significance of these changes in relation to the promotion of mammary carcinogenesis has not been established. Further experiments are therefore required to correlate these changes to the development of mammary tumors, and to explore the effects of high fat diet on the metabolic activities of the mammary gland.

It is now possible to separate the mammary epithelial cells from the surrounding fat cells by collagenase treatment (Janss and Moon, 1970). This provides an opportunity to study the mammary epithelial cells and the fat cells separately. The importance of adipose tissue to the growth of transplanted normal mammary gland has been demonstrated by Hoshino (1962, 1964) in mice that regeneration of mammary transplants was
determined mainly by the amount of adipose tissue at the site of transplantation. The hypothesis that hyperplastic alveolar nodules (HANs) represent the precancerous stage of mammary tissue in rats and in mice have been discussed earlier in this discussion. Recently Bartley, McGrath and Abraham (1971) found that HANs have lost the ability to make the unique pattern of fatty acids characteristic of normal mammary gland, that is, the synthesis of predominantly medium-chain fatty acids. The above workers regarded this deletion a deviation from normal to neoplasia since the pattern of fatty acid synthesis in HANs was more like that of mammary adenocarcinomas than of normal mammary tissue in mice. Therefore, it will be of some interest to study the ability of the mammary epithelial cells to metabolize fat under different dietary conditions.

Ovariectomy performed 30 days before carcinogen administration has been shown by a number of investigators (Dao, 1962a; Talwalker et al, 1964; Welsch et al, 1968; Beuving, 1969) to inhibit totally the induction of mammary tumors in rats. However, if the same operation was performed later than 30 days before carcinogen administration it only reduced mammary tumor incidence and lengthened the latent period. The results indicate that the presence of sufficient amounts of estrogen is essential for mammary tumor induction. Ovariectomy has been shown to lower plasma prolactin level significantly. (Pearson, Llerena, Llerena, Malina and Butler, 1969).

Administration of estradiol has been shown to have dual effect on the development of mammary tumors in rats (Buggins et al, 1959a; Kim et al, 1963). Small doses of estradiol (0.1-3 μg) stimulated and large doses (20 μg or more) inhibited the development of mammary tumors.
Chen and Meites (1970) found that small doses of estradiol increased markedly the level of plasma prolactin but large doses of estradiol tended to retain the prolactin in the pituitary. A positive correlation was found by Kim et al (1963) between the level of plasma prolactin and changes in mammary tumor size. The effect of prolactin alone on chemically induced mammary carcinogenesis has been controversial since contradictory results were reported in the literature (Klaiber et al, 1969; Welsch et al, 1970b; Nagasawa and Yanai, 1970). It is very likely that estrogen and prolactin act synergistically to promote mammary carcinogenesis (Dao, 1969a; Boot, 1970).

In view of the above evidence, Carroll and Khor (1970) suggested that high fat diet may alter the hormonal environment of mammary tissue by changing the distribution and metabolism of steroid sex hormones. To test this hypothesis, tritiated estradiol-17β was injected into female rats fed on high and low corn oil diets and the distribution of radioactivity was measured in several tissues. The results (Figs. 11-13) indicate that, in general, rats on low corn oil diet tended to accumulate and retain more radioactivity in their tissues than those on high corn oil diet. The difference in radioactivity was greater in the uterus than that of the other tissues between the two groups. Within the same group, the radioactivity in the pituitary and the uterus was very much higher than that of the other tissues when comparison was made on wet weight basis. This is in agreement with the findings of others (Jensen and Jacobson, 1962; Eisenfeld and Axelrod, 1965, 1968; Sander, 1968) who reported selective uptake of tritiated estradiol in the pituitary and uterus of rats fed on laboratory chow diet. It has repeatedly confirmed that the unchanged labelled estradiol accounted for over 90% of the
radioactivity accumulated in the pituitary, uterus and mammary gland (Jensen and Jacobson, 1962; King et al, 1965b; Puca and Bresciani, 1968). Therefore, the observed differences in the uptake of radioactivity between the two groups could be due to differences in metabolism of estradiol in the liver. A preliminary experiment carried out to test this possibility showed that one hour after injection of estradiol-4-\textsuperscript{14}C, the liver of rats on high corn oil diet converted significantly more (P<0.001) of this steroid to its water soluble metabolites than that of rats on low corn oil diet (Table XIII). Although the components of the chloroform fraction were not characterized in our study, it was believed, based on the information in the literature, that this fraction contained only free estrogens. Thus, it appears that differences in the level of radioactivity in tissues of the two dietary groups after injection of tritiated estradiol may be due to differences in metabolism of this steroid in the liver.

It is interesting to note that there are differences in metabolism of estradiol, one of the important steroid hormones known to be involved in mammary carcinogenesis. It is possible that the enzymes responsible for the metabolism of estrogens may be stimulated in rats by feeding high corn oil diet. Further experiments are needed to check this possibility.

The significance of the findings on the distribution and metabolism of labelled estradiol in relation to promotion of mammary carcinogenesis is not certain at this moment because of two reasons. Firstly, corn oil was the only dietary fat tested in this experiment, and secondly it was found in the long-term experiments reported earlier in this thesis that saturated fats and oils had, in general, smaller promoting
effect on DMBA-induced mammary carcinogenesis than unsaturated fats and oils. Therefore, in future experiments, other saturated and unsaturated fats and oils should be tested for their ability to enhance the metabolism of labelled estradiol in liver. Such information will decide whether or not differences in metabolism of estradiol are related to promotion of mammary carcinogenesis by high fat diet.

Progesterone is present in the circulation of nonpregnant rats in very small quantity (Feder, Resko and Goy, 1968). Administration of progesterone after the treatment of chemical carcinogens (Huggins et al., 1959a; Huggins and Yang, 1962; Dao, 1964a; Jabara, 1967) and pregnancy developing after carcinogen treatment (Dao and Sutherland, 1959; Dao et al., 1960; Huggins et al., 1962; McCormick and Moon, 1965) have been shown to promote mammary carcinogenesis in the rat. In view of these facts, the distribution of tritiated progesterone in female rats fed on high and low corn oil diets was studied. The results (Fig. 14) indicate that there is not much difference in the accumulation of radioactivity in various tissues obtained from rats on two different diets. There is also no selective accumulation of radioactivity in the pituitary and uterus in each group and the liver appears to have the highest radioactivity. This is in accordance with the findings of other investigators (Lawson and Pearlman, 1964; Laumas and Farooq, 1966; Seiki, Higashida, Imanishi, Miyamoto, Kitagawa and Kotani, 1968) who also found no selective uptake of tritiated progesterone in the pituitary and uterus of female rats fed on laboratory chow diet. However, the dose of progesterone used in our study was several times smaller than that used by other workers.

It is very interesting to note that high corn oil diet has
considerable effects on the distribution and metabolism of estradiol and has no effect on the distribution of progesterone in female rats. Studying the uptake of simultaneously administered $^3$H-estradiol and $^{14}$C-progesterone by DMBA-induced mammary tumors in female rats, Mobbs (1968a) noticed that progesterone was not taken up and retained by hormone-responsive adenocarcinomas to the same extent as estradiol. Mobbs (1968a) suggested that the mechanism of action of progesterone was different from that of estradiol and did not involve retention by the target tissue. Chen and Meites (1970) reported that progesterone was very much less effective in increasing plasma prolactin level compared to estradiol and a very large of progesterone (10 mg. daily) increased moderately the level of plasma prolactin in female rats. Hence, Chen and Meites (1970) thought that it was not likely that the tumor-promoting activity of progesterone in pregnancy and pseudopregnancy was mediated through the release of prolactin from the pituitary. Progesterone may have other mode of action.

Analysis of the composition of plasma lipids showed that female rats on low corn oil diet had significantly higher plasma triglyceride level and low plasma free fatty acid level than those on high corn oil diet. However, there was not much difference in the level of other plasma lipids between the two groups. In our semisynthetic diets (Appendix I) the protein content is kept fairly constant and a decrease in the content of fat is compensated by an increase in the content of carbohydrate (i.e. dextrose). Therefore, the low corn oil diet is actually a high carbohydrate, low fat diet. Such a diet has been observed to increase plasma triglyceride level and to lower plasma free fatty acid level in man (Gordon and Cherkes, 1956; Nikkila, 1969).
High carbohydrate, low fat diet has also been found to increase plasma triglyceride level in rats. Some carbohydrates appear to have a more striking effect than others and glucose causes only a moderate increase in plasma triglyceride level in rats (Nikkila and Ojala, 1965; Bar-On and Stein, 1968). The higher plasma triglyceride level and lower plasma free fatty acid level are, therefore, in agreement with the findings reported in the literature (Gordon and Cherkes, 1956; Bar-On and Stein, 1968; Nikkila and Ojala, 1965; Nestel, Carroll and Havebstein, 1970).

The significance of these findings in relation to promotion of mammary tumor production in DMBA-treated rats is not known. However, diabetes, a condition often associated with hyperglyceridemia, induced in mammary tumor-bearing rats caused rapid regression in 90% of the existing tumors. On the other hand, injection of insulin, which lowered plasma triglyceride level, markedly stimulated the growth of existing mammary tumors (Heuson and Legros, 1970). It is interesting to note that the growth of transplanted solid tumors is associated with increases of plasma free fatty acid level in rats. (Buzzi, Garattini and Guaitani, 1968).
SUMMARY AND CONCLUSIONS

(1). Present studies confirm the previous finding that a semisynthetic diet containing 20% corn oil enhanced the yield of mammary tumors induced in female Sprague-Dawley rats by 7,12-dimethylbenz-(a)anthracene (DMBA) compared to a similar diet containing 0.5% corn oil. Additional information on the effects of intermediate levels of dietary corn oil are presented. A 10% corn oil diet had the same promoting effect on DMBA-induced mammary carcinogenesis as a similar diet containing 20% corn oil; whereas a 5% corn oil diet gave low tumor yield similar to that obtained with a 0.5% corn oil diet.

(2). For the first time, a series of fats and oils was tested for their tumor-promoting property in rats under the same experimental conditions by feeding them at the level of 20% by weight of the diet. It was found that there was an apparent trend toward increased tumor yields with increasing unsaturation of the dietary fats. However, there was not much different in the percentage of rats bearing mammary tumors among the different groups. Rapeseed oil was an obvious exception and experiments are in progress to determine the cause of this unusual response.

(3). In our semisynthetic diets, the protein content is kept fairly constant and a decrease in the content of fat is compensated by an increase in the content of carbohydrate and vice versa. Therefore,
there is a possibility that the enhancing effect of high fat diet may be due to changes in the level of dietary carbohydrate. However, the fact that diets containing unsaturated fats gave higher tumor yields than those containing saturated fats suggested that the effect was due to dietary fat rather than due to dietary carbohydrate since the high fat diets differ only in the nature of fat used.

(4). The promoting effect of high corn oil (20% by weight) diet could be seen with mammary tumors induced by a single intravenous injection of DMBA-fat emulsion indicating that the promoting effect of high corn oil diet was not associated with the route of administration of the carcinogen.

(5). Transferring rats from low corn oil (5% by weight) diet to high corn oil (20% by weight) diet one week after DMBA administration produced a significantly higher tumor yield and a shorter latent period, compared to control group fed on low corn oil diet. When the feeding of high corn oil diet was delayed for two weeks after DMBA administration, the tumor yield was decreased slightly and the latent period was prolonged slightly, but the tumor yield was still considerably higher than that of the control. However, when the feeding of high corn oil diet was delayed for four weeks after DMBA administration, most of the promoting effect of high corn oil diet was eliminated and the result was similar to that of the control.

(6). Transferring female rats from high corn oil (20% by weight) diet to low corn oil (5% by weight) diet one, two and four weeks after DMBA administration inhibited the development of mammary tumors, as a result of which the percentage of rats with tumors and the final
tumor yield was lower and the latent period was longer than of the control fed on high corn oil diet. However, low corn oil diet fed to rats beginning one week after DMBA administration appeared to have a greater inhibitory effect on tumor development than the same diet fed either beginning at two or four weeks after DMBA administration.

(7). Feeding low corn oil diet to female rats beginning two months after DMBA administration did not appear to affect the growth of well-established mammary tumors that developed before the change of diet nor did it appear to affect the final tumor yield, compared to controls on high corn oil diet.

(8). From the above results it was concluded that high corn oil diet affected the development of mammary tumors but did not appear to have any effect on the growth and maintenance of mammary tumors. The period immediately after the initiation and before the appearance of palpable tumors was probably most susceptible to alteration of the level of fat in the diet.

(9). Female rats fed on low corn oil (5% by weight) diet consistently accumulated more radioactivity of estradiol-6,7-\(^3\)H in several tissues at different intervals after injection, compared to those on high corn oil (20% by weight) diet. The greatest difference in radioactivity was found in the uterus of rats on the two diets. The experiments were carried out in 50-60 and 90-100 day old rats. Results obtained with 90-100 day old rats were more variable than those obtained with 50-60 day old rats.

(10). The liver of female rats fed on high corn oil (20% corn oil) diet converted significantly more estradiol-4-\(^14\)C to its water soluble
metabolites than the liver of female rats fed on low corn oil (5% by weight) diet one hour after intravenous injection. This could account for the differences in uptake of radioactivity between the two dietary groups. However, the significance of this finding in relation to promotion of mammary carcinogenesis by high fat diet cannot be assessed at this moment. Further experiments on the metabolism of $^{14}$C-labelled estradiol in the liver of rats fed on diets containing other saturated and unsaturated fats are needed.

(11). There was not much difference in the uptake of progesterone-$1,2^{-3}$H in 90-100 day old female rats fed on high corn oil (20% by weight) diet and low corn oil (5% by weight) diet at different intervals after intravenous injections. There was also no selective uptake of radioactivity in the pituitary and the uterus.

(12). Analysis of the lipid composition of plasma obtained from female rats fed on high corn oil (20% by weight) and low corn oil (5% by weight) diets showed no significant difference in total lipids, phospholipids, neutral lipids and cholesterol contents in the plasma between the two groups. However, rats on low corn oil diet had significantly higher level of plasma triglycerides and significantly lower level of plasma free fatty acids than those on high corn oil diet.
REFERENCES


Cancer Res. 1: 807-814.


Bird, C. C., Crawford, A. M., Currie, A. R. and Fiona Stirling, B. (
1970). Protection from the embryopathic effects of 7-hydroxymethyl- 
12-methylbenz(α)anthracene by 2-methyl-1,2-bis-(3-pyridyl)-1-pro-
panone (Metopirone, Ciba) and -diethylaminoethylidiphenyl-n-pro-

free fatty acids during the growth of various experimental tumors. 

Bock, F. G. and Dao, T. L. (1961). Factors affecting the polynuclear hy-


Boyland, E. and Sims, P. (1965a). The metabolism of 7,12-dimethylbenz 
(α)anthracene by rat liver homogenates. Biochem J. 95: 780- 
787.

necrosis and cancer with metabolites of 7,12-dimethylbenz(α)an-

Boyland, E. and Sims, P. (1967a). The effect of pretreatment with adre-
nal- protecting compounds on the metabolism of 7,12-dimethylbenz-
z(α)anthracene and related compounds by rat liver homogenates. 

Boyland, E. and Sims, P. (1967b). The carcinogenic activities in mice 
of compounds related to benz(α)anthracene. Int. J. Cancer 2:
500-504.


Endocrinology. 86: 503-505.


Dao, T. L. (1964b). Some consideration of molecular structure of polynuclear hydrocarbons and inhibition of adrenal necrosis in


nodules transplanted into gland-free mammary fat pad C3H
digestibility of rapeseed oil in the rat. J. Nutr. 35: 295-
300.
p. 222.
De Waard, F. (1969). The epidemiology of breast cancer: Review and
Dilley, W. J. and Nandi, S. (1968). Rat mammary gland differentiation
Downey, R. K., Craig, B. M. and Youngs, C. G. (1969). Breeding Rape-
seed for oil and meal quality. J. Amer. Oil Chemists Soc.
46: 121-123.
dietary fat and carbohydrate on diethylstilbestrol-induced
Dunning, W. F. and Curtis, M. R. (1956). The respective role of long-
evity and genetic specificity in the occurrence of spontan-
eous tumors in hybrids between two inbred lines of rats.
Cancer Res. 6: 61-68.
Durbin, P. M. H., Williams, N. J., Arnold, J. S. (1966). Development
of spontaneous mammary tumors over the life span of the
female Charles River (Sprague-Dawley) rats. The influence
ovariectomy, thyroidectomy and adrenalectomy-ovariectomy.
Cancer Res. 26: 400-411.


Flesher, J. W., Soedigo, S. and Kelly, D. R. (1967). Synthesis of metabolites of 7,12-dimethylbenz(α)anthracene, 4-hydroxy-7, 12-dimethylbenz(α)anthracene, 7-hydroxymethyl-12-methyl-


Jellinck, P. H. and Smith, G. (1969). Metabolism of 7-hydroxymethyl-


London, Ontario.


King, R. J. B. and Gordon, J. (1968). An attempt to isolate an estra-
diol receptor from nuclei by adsorption on estradiol-17β. J. Endocr. 40: 195-204.


McCann, S. M. and Dhariwal, A. P. S. (1966). Hypothalamic releasing factors and the neurovascular link between the brain and


Ulland, B., Finkelstein, M., Weiburger, E. K., Rice, J. M. and Weiburger,


Weiburger, J. H., Weiburger, E. K., Griswold, D. P. Jr. and Casey, A. E.


APPENDIX I

Composition of Semisynthetic Diets*

<table>
<thead>
<tr>
<th></th>
<th>0.5% Fat</th>
<th>5% Fat</th>
<th>10% Fat</th>
<th>20% Fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>18</td>
<td>19</td>
<td>20.5</td>
<td>23</td>
</tr>
<tr>
<td>Dextrose</td>
<td>72</td>
<td>66.5</td>
<td>59.5</td>
<td>46</td>
</tr>
<tr>
<td>Fat</td>
<td>0.5</td>
<td>5</td>
<td>10.0</td>
<td>20</td>
</tr>
<tr>
<td>Salt Mixture</td>
<td>4</td>
<td>4</td>
<td>4.5</td>
<td>5</td>
</tr>
<tr>
<td>(Phillips-Hart)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Celluflour</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

*Adequate vitamins supplements were added to these diets (Carroll and Khor, 1970).*