CHAPTER - VIII

General Discussion
Cancer is a disorderly growth of cells which invade adjacent tissues and spread by the lymphatics and blood vessels to other parts of the body. The unchecked growth of a cancer ultimately leads to death of the host. Any agent which initiates the growth of a cancer is called a carcinogen. Many chemicals, of natural and of industrial origin, have been implicated as carcinogens. 3-methylcholanthrene, a polycyclic aromatic hydrocarbon, was first synthesized in 1934 by Wieland and Dana, and its carcinogenic properties demonstrated by Cook and Haslewood (1934). This hydrocarbon is one of the most powerful synthetic carcinogens known. It has been shown to produce mammary, sebaceous and cutaneous neoplasms, leukemia (Greenstein et al., 1962), hepatomas, pulmonary adenomas, forestomach papillomas, lymphocytic neoplasms and adenocarcinomas of the large intestine (Klein, 1963). The present investigation showed that a single intraperitoneal injection of 3-methylcholanthrene (10 mg. in 2.0 ml. castor oil) caused cancerous changes in the liver and mesentery (figs. 19a, 19c) accompanied by extensive proliferative changes in other organs.
Conney and Burns (1959) observed for the first time that 3-methylcholanthrene markedly stimulated the excretion of ascorbic acid in rat urine. In normal circumstances the urinary ascorbic acid level in rats remains practically constant. A number of other compounds, possessing totally unrelated chemical and pharmacological characteristics, also enhance the urinary excretion of ascorbic acid. The effect of the carcinogenic hydrocarbon is, however, much more potent and long-lasting in comparison to other compounds. While other drugs registered their effect by the first or second day a single intraperitoneal injection of 3-methylcholanthrene was observed to show no effects on the urinary excretion of ascorbic acid for the first two days (Conney and Burns, 1959; Burns et al., 1960). These workers reported that the excretion then gradually rose until it reached a peak, at 50–75 times the normal level, by about 6 to 8 days. Boyland and Grover (1961), in a similar experiment, reported a much smaller increase (≈3 fold) though the general pattern of excretion was similar to that reported by Conney and Burns (1959). In the present study also the carcinogen was observed to exert no
effect for the first two days after an intraperitoneal injection of 3-methylcholanthrene, a peak in the urinary excretion of ascorbic acid was reached by the eighth day. The mean peak value was about five times the pre-injection level. A different pattern of the urinary excretion of ascorbic acid was noted when 3-methylcholanthrene was administered along with ascorbic acid. The peak urinary excretion of ascorbic acid in this group of animals was found to be similar to urinary excretion of ascorbic acid in the group of animals receiving ascorbic acid alone. Both these groups excreted a maximum amount of ascorbic acid by the second day, but the mean value for the carcinogen + ascorbic acid treated group was twice the mean value of the ascorbic acid treated group. While the urinary excretion of the latter group fell slowly back to normal levels, that of the former remained elevated. This seems to suggest that the simultaneous administration of ascorbic acid increases the effectiveness for the renal clearance of ascorbic acid.

The urinary excretion of ascorbic acid fell back to pre-injection values in the 3-methylcholanthrene treated animals by the 10th day, while it
remained elevated in the 3-methylcholanthrene + ascorbic acid group. Conney and Burns (1959) and Burns et al. (1960) had however reported that the level of urinary ascorbic acid remained above the value observed before treatment even after 18 days.

The reason for the enhanced excretion has been attributed by Conney and Burns (1959) to be due to a stimulation of ascorbic acid synthesis. Burns et al. (1960) suggested that this increase in biosynthesis was due to an increased metabolism of glucose through the glucuronic acid pathway and Evans and her colleagues (1960) demonstrated that the enhancement of ascorbic acid excretion involved an increased rate of formation of the vitamin and of its precursors D-glucuronate and gulonate.

Conney et al. (1956, 1957) had earlier observed that the carcinogenic hydrocarbons were extremely potent in inducing the synthesis of several liver microsomal enzymes which metabolize foreign compounds. The enzyme system concerned in the biosynthesis of ascorbic acid is located in the liver microsomes in mammals (Chatterjee et al., 1961). Parke (1968) observed that the activation of the microsomal drug-metabo-
lizing enzymes by 3-methylcholanthrene occurs before
the increase in ascorbic acid synthesis, and suggested
that these two reactions of the carcinogen did not
necessarily involve the same mechanism.

Since 3-methylcholanthrene was observed
to stimulate glucuronate formation, Touster and
Hollman (1961) suggested that the stimulation occurred
at a step preceding this intermediate. They studied
the activities of the enzymes, UDPG dehydrogenase
which catalyzes the formation of UDPG to UDP-glucuro-
nic acid, and UDP-glucuronic acid pyrophosphatase
which catalyzes the formation of glucuronic acid from
UDPG-glucuronic acid, in the livers of normal and 3-
methylcholanthrene treated rats. No significant chan-
ges in the activities of these two enzymes from con-
trol levels was observed in the treated animals
(Touster and Hollman, 1961). Conney et al. (1961) obse-
ved an increased ability in 3-methylcholanthrene
treated rats to metabolise D-galactose-1-C^{14} to labe-
lied L-gulonic and L-ascorbic acids. Since no increa-
se was observed in the excretion of D-glucuronic acid,
simultaneously, Conney et al. (1961) suggested that 3-
methylcholanthrene may stimulate the further metabolism
of D-glucuronic acid. The evidence produced so far does not confirm the direct effect of 3-methylcholanthrene on the glucuronic acid cycle, and the mechanism of action of the carcinogen on ascorbic acid synthesis remains obscure.

In the present investigation, the effect of 3-methylcholanthrene on hepatic ascorbic acid concentration was studied. It was observed (table VII) that intraperitoneal injection of a 10 mg. dose of 3-methylcholanthrene in castor oil led to a marked decrease in liver ascorbic acid content (P < .05) 12 days after injection, compared with animals treated with castor oil alone. The ascorbic acid content of the liver in the 3-methylcholanthrene injected animals was reduced to about one-tenth of the level observed in untreated controls. The hepatic ascorbic acid concentration was also decreased significantly in animals injected with 10 mg. of 3-methylcholanthrene and 200 mg. of ascorbic acid simultaneously. No significant difference was observed between this group and the group treated with 3-methylcholanthrene alone. These findings suggest a mechanism other than increase in synthesis of ascorbic acid is involved
in high urinary excretion of ascorbic acid after administration of 3-methylcholanthrene.

The histopathological changes occurring in the liver of rats injected with 3-methylcholanthrene was seen to be characteristic of premalignant lesions. No such lesions were observed in sections of liver taken from rats injected with 3-methylcholanthrene and ascorbic acid. This might be due to the anticarcinogenic property which has been attributed to ascorbic acid by Warren (1943), Pipkin et al. (1969), and Schlegel et al. (1969), or it could also be due to individual variations on the part of the animals. The exact time of histopathological changes after administration of 3-methylcholanthrene could not be ascertained at this stage nor is it possible to state whether the precancerous change occurs concurrently with the rise in ascorbic acid excretion and depletion of hepatic ascorbic acid.

The effect of 10 mg. 3-methylcholanthrene in castor oil, 10 mg. of 3-methylcholanthrene + 200 mg. of ascorbic acid in castor oil, and castor oil alone on adrenal and blood ascorbic acid content was studied. It was seen that the concentration of ascorbic acid in the adrenal glands and blood of these
animals did not differ from the control values (tables VIII, IX), though the liver ascorbic acid in these animals was found to be decreased.

The results of this investigation differed from that of other workers (Kennaway et al., 1944; Daff et al., 1948) who reported an increase in the hepatic ascorbic acid concentration in mice after a single injection of a variety of carcinogens including 3-methylcholanthrene, however, earlier studies carried out by Boyland and Mawson (1938) did not show any increases in hepatic ascorbic acid with smaller doses of carcinogens. Reduction in the hepatic ascorbic acid content noted in the present investigation after administration of 3-methylcholanthrene might therefore be explained in terms of the amount of methylcholanthrene administered. However, this hypothesis is to be tested with further experiments with varying amount of methylcholanthrene. The finding that a single intraperitoneal injection of 3-methylcholanthrene does not alter adrenal and blood ascorbic acid levels is in agreement with the findings of Dao et al. (1963). These workers had suggested that the stimulatory effect of 3-methylcholanthrene on adrenal ascorbic acid synthesis may be associated
with the inhibition of corticosterone in the adrenal cortex.

The present study indicated that after an intramuscular injection of ascorbic acid the vitamin concentration was increased in the blood and liver but was unaffected in the adrenals. This agrees with the pattern observed by Sarkar and Goswami (1975). Martin (1961) had reported that ascorbic acid was quickly removed from the serum and transferred to tissues such as adrenal and liver.

The synthesis of ascorbic acid from glucose is one of the main pathways available for glucose metabolism in rats. The liberation of glucuronic acid from UDP-glucuronic acid involves two stages.

$$\text{Glucose} \rightarrow \text{Glucose-6-P} \rightarrow \text{Glucose-1-P}$$

The second stage is a reaction involving a phosphatase. The regeneration of glucose from glucose-6-phosphate involves the enzyme glucose-6-phosphatase. It was suggested that 3-methylcholanthrene may influence the biosynthesis of ascorbic acid in any one of...
the steps involving phosphatase. Any changes in the activity of glucose-6-phosphatase would result in disturbance in the availability of glucose as substrate for the synthesis of ascorbic acid.

The rat liver and kidney glucose-6-phosphatase is bound to the microsomes (Hers, 1964), however, in rat liver one fourth of the activity is found in the mitochondria (Dixon and Webb, 1967). Its specificity is far from absolute and it hydrolyses a wide variety of primary phosphate esters (Hers, 1964). The microsomal glucose-6-phosphatase of vertebrate liver has been shown to have the ability to synthesise as well as hydrolyse glucose-6-phosphate under suitable conditions (Stetten and Goldsmith, 1976; Stetten and Taft, 1964; Nordlie, 1974).

A single intraperitoneal injection of 3-methylcholanthrene increases the level of several rat liver microsomal enzyme systems (Conney et al., 1956; Gelboin et al., 1958; Conney et al., 1959) while other enzymes either remain unchanged or are depressed (Conney et al., 1959). In the present investigation it was observed that glucose-6-phosphatase activities in the livers of rats remained unaffected 12 days after...
intraperitoneal administration of i) 10 mg. of 3-methylcholanthrene in 2.0 ml. castor oil ii) 10 mg. of 3-methylcholanthrene and 200 mg. of ascorbic acid in 2.0 ml. castor oil iii) 200 mg. of ascorbic acid and iv) 2.0 ml. of castor oil (tables XIV & XII).

The activity of liver glucose-6-phosphatase has been reported to be decreased in rats treated with 4-dimethylaminoazobenzene (Weber and Cantero, 1983) and diethylnitrosamine (Elms et al., 1977; Kil'dema et al., 1977), while glucose-6-phosphatase activity was found to be totally absent in a number of tumours (Weber and Cantero, 1955). No changes were noted in the present investigation after administration of 3-methylcholanthrene, indicating that the carcinogen does not affect the phosphatase activities, 12 days after administration. Since ascorbic acid excretion is markedly raised in rats, injected with 3-methylcholanthrene, during this period the study seems to suggest that 3-methylcholanthrene does not act on ascorbic acid synthesis by influencing the activity of glucose-6-phosphatase. However, it may be pointed out that the peak urinary excretion of ascorbic acid was noted on the 8th day after administration of methylcholanthrene and as such successive
estimations of hepatic glucose-6-phosphatase and hepatic ascorbic acid content at different times after administration of methylcholanthrene might be useful for a definite conclusion.

A possible relationship between enzyme induction and carcinogenesis has been studied by many workers. Conney (1967) observed that carcinogenic hydrocarbons stimulated a limited number of enzymic reactions when compared to non-carcinogenic drugs. Cramer et al. (1960) and Conney et al. (1956) demonstrated that enzyme induction by 3-methylcholanthrene blocked the carcinogenic effects of amino-azo dyes and 2-acetylaminofluorene.

Gelboin and Blackburn (1964) felt that the 3-methylcholanthrene induced changes in the microsome enzyme activities were due to an alteration in the protein synthetic apparatus and in the expression of specific genetic information.

Touster and Hollman (1961) observed that the enhancement in ascorbic acid excreted which followed the administration of 3-methylcholanthrene was blocked by the simultaneous feeding of ethionine. Administration of methionine was seen to nullify the
ethionine block. This led Touster and Hollman (1961) to postulate that extra protein synthesis was involved in the rise in ascorbic acid excretion. In the present series, however, no changes in the protein content, per 100 mg. of liver tissue, was observed in rats 12 days after a single intraperitoneal injection of 10 mg. of 3-methylcholanthrene, and 10 mg. of the carcinogen with 200 mg. of ascorbic acid (table XIII). The administration of ascorbic acid alone also did not affect the protein concentration in liver.

3-methylcholanthrene has been found to cause little or no increase in the amount of microsomal protein per gram of liver, but was observed to stimulate liver growth and the synthesis of total liver protein (Conney et al. 1956; Aruoma et al. 1961; Conney and Gilman, 1963).

Ascorbic acid can be considered to be an intermediate in carbohydrate metabolism as it is known to be synthesised from glucose (Jackel et al., 1950; Horowits et al., 1952), and to be converted back to glucose (Dayton et al., 1959). Deficiency of ascorbic acid leads to disturbances in carbohydrate metabolism, and a lowering of liver glycogen levels have
been reported in scurvy (Banerjee, 1943; Murray and Morgan, 1946; Banerjee et al., 1948).

One of the main features of tumour cells is their high rate of aerobic and anaerobic glycolysis. Orr and Stickland (1941) pointed out that the substrate for glycolysis in the normal liver is glycogen, while in the rat hepatoma it is glucose. Olson (1951) observed that the hepatoma diverted glucose from glycogen synthesis into the glycolytic pathway. A number of studies revealed that the glycogen content of tumours was lower than the normal levels observed in liver (Greenstein, 1943; Le Page, 1948; Goranson, 1955; Ball et al., 1957). Spain and Griffin (1956) found that the cells of the central zone of the liver lost their capacity to retain or synthesise glycogen, a few days after azo dye feeding.

It was observed (table XVIII) in the present investigation that there was a significant reduction in liver glycogen levels in rats 12 days after injection of 10 mg. of 3-methylcholanthrene, when compared with control levels (P<0.05). The glycogen levels in another group of animals, which received 10 mg. of 3-methylcholanthrene simultaneously with 200 mg. of ascorbic acid, were found not to differ from
the control values \( (P > 0.1) \). This seemed to suggest that administration of ascorbic acid along with 3-methylcholanthrene counteracts the glycogenolytic effect of the carcinogen.

Sarkar and Goswami (1975) had earlier noted that liver glycogen was markedly decreased in rats killed half an hour after administration of ascorbic acid. In the present series, the animals were sacrificed an hour after injection of ascorbic acid, and no significant change was noted from the control levels and concluded that the glycolytic effect of ascorbic acid is very short lived and transitional.

Ascorbic acid is involved in the metabolism of many of the amino acids. It is responsible for the complete oxidation of tyrosine (La Du and Zannoni, 1961), and in hydroxylation of phenylalanine, and proline (Gould and Woessner, 1957; Napson, 1967) and tryptophan (Cooper, 1961).

An alteration of the free amino acid pattern more particularly variation in the levels of tyrosine, phenylalanine and proline, was expected to result on administration of ascorbic acid. In the present investigation a very significant increase in
free leucine and proline (P < 0.005) was observed in the serum of rats killed an hour after injection of 200 mg. of ascorbic acid (table XIX). A slight increase was noted in the mean levels of free glutamine, alanine, valine, glutamic acid and phenylalanine was observed, while the levels of the other free amino acids were unaffected.

Each amino acid in the plasma is in equilibrium with an intracellular pool of the same amino acid. Raw material is taken from this pool for both catabolic and anabolic purposes. In malignancy, the tumour accumulates nitrogen at the expense of the host and Mider (1951) described tumours as "nitrogen traps".

Studies carried out by Roberts and Frankel (1949) on epidermis of mice treated with 3-methylcholantrhene exhibited a greater concentration of free amino acids than normal skin, than the resulting squamous cell carcinoma. White et al. (1954) had reported an increased level of free glutamic acid in the plasma of rats treated with 3-methylcholantrhene.

In the present investigation a definite disturbance was noted in the free amino acids in the serum of rats injected with 10 mg. of 3-methylcholantrhene, and also in the animals injected with 200 mg.
of ascorbic acid along with 10 mg. of 3-methylcholan-
threne, 12 days after administration of the compounds.

The levels of free glycine and alanine were found to be significantly elevated \((P < 0.005)\) above the control levels in the 3-methylcholanthrene treated group (table XIX). The levels of the other glucogenic amino acids, glutamic acid, histidine, valine and proline also showed a sharp increase in the mean values; the difference, however, was statistically not very significant. A rise was noted in the amino acids, leucine, phenylalanine and tyrosine, the rise being statistically significant \((P < 0.01)\) in the case of leucine.

In the case of the animals treated with 3-methylcholanthrene and ascorbic acid simultaneously, the mean values of the free amino acids in serum were observed to rise but was not significant for any of the amino acids. This suggests that the administration of ascorbic acid along with the carcinogen in some way mitigates the effect of the carcinogen.

Aside from a few special pathways the catabolism of most amino acids commences with a deami-
nation. The carbon skeleton of most of the amino acids
then readily participates in gluconeogenesis; while others give rise to acetoacetic acid and acetyl co-
acetyl co-

c-enzyme A. Any increase in the concentration of free amino acids in blood might result in an increase in gluconeogenesis.

If high urinary excretion of ascorbic acid after methylicholangthrene administration is accepted to be a result of increased ascorbic acid synthesis, the present investigation rather suggests that increased levels of glucogenic amino acids make glucose available for the purpose.

Ascorbic acid has been suggested for the treatment of various diseases which are not directly related to a deficiency of the vitamin. Very high doses of ascorbic acid have been shown to inhibit the carcinogenic activities of many substances and to prevent proliferation of the malignancy (Kennaway et al. 1955; Banade et al. 1969; Cameron and Campbell, 1974). Schlegel et al. (1969) suggested that regular administration of a high dose of ascorbic acid to smokers and patients with recurrent bladder tumours reduced the risk of carcinoma.

The anticarcinogenic property has been attributed to its ability to prevent spontaneous oxi-
dation of carcinogens like 3-hydroxyanthranilic acid (Schlegel et al. 1969), or to its role in detoxifying carcinogens like anthracene and 3,4-benzyrene (Warren, 1943). It has also been noted in the course of the present investigation that simultaneous administration of a high dose of ascorbic acid with the carcinogenic hydrocarbon, 3-methylcholanthrene, to rats inhibited the effect of the carcinogen in inducing cancerous changes in the liver, in liver glycogenolysis, as well as in raising the level of the free amino acids in the serum.

In the present investigation it was attempted to study the relationship of ascorbic acid with nasopharyngeal carcinoma. Bodansky et al. (1951) had observed significantly lower values of plasma ascorbic acid in a group of cancer patients when compared with healthy controls. In the present series no significant variation in the blood ascorbic acid levels was observed in the group of patients before and after treatment when compared with the mean levels of the healthy control group (P > 0.1 and >0.05 respectively). The frequency distribution patterns of blood ascorbic acid was, however, observed to be different
in the three groups (fig.45) with 17% of the patients having levels of blood ascorbic acid below the lower limit observed for the normal group.

Watson (1943) and Mcl (1956) had hypothesized that tumour cells have a higher requirement of ascorbic acid, resulting in an increased consumption of the vitamin and a lowered urinary excretion. The present findings do not support this hypothesis as the pattern of urinary excretion was not found to differ among the group of patients before and after treatment and the control group. No clear cut relationship between mesopharyngeal carcinoma and ascorbic acid was seen in this investigation though a disturbance in ascorbic acid levels in the blood of carcinoma patients was noted. Various hypotheses have been put forward to explain the anticarcinogenic property of ascorbic acid, but none have been accepted or confirmed beyond doubt.

Metabolism of amino acids in the cells is regulated by many factors including enzymes and co-enzymes. Ascorbic acid acting as coenzyme influences the complete metabolism of some amino acids. It was seen in the earlier part of the investigation that
levels of ascorbic acid in tissues and urine are altered in malignancy induced by 3-methylcholanthrene, and in mesopharyngeal carcinoma. The effect of mesopharyngeal carcinoma on the levels of free amino acids in serum was studied.

It was observed that, in the patient in the present series, the levels of all the free amino acids in serum were significantly decreased from the values observed in the normal healthy group. Only the levels of lysine and histidine showed no significant change. The decrease is most marked in the case of glutamine, alanine and proline (P < .005) and quite significant (P < .02) for glycine, glutamic acid, tyrosine, valine, phenylalanine and leucine. Earlier studies in cancer patients had reported a rise in plasma glutamic acid (Mo Henry, 1955; Rouser et al., 1962; Iyer, 1958) and lowered levels of glutamine and other amino acids (Rouser et al., 1962; Iyer, 1958). The levels of the serum free amino acids in the cancer patients were not significantly altered on completion of treatment with deep radiation therapy (table XXXIV). The exceptions were proline, and lysine and histidine; the levels of these free amino acids in the serum were lower than the pre-treatment values (P < .002) and P < .02 respectively).
The mean amino acid nitrogen of whole blood in the patients studied was observed to be significantly elevated \( (P < 0.005) \) above the normal values. The difference persisted even after completion of treatment with deep radiation therapy \( (P < .005) \) and endoxan \( (P < .05) \). Earlier studies on the amino acid nitrogen content of blood had been carried out mostly in leukemias and the results obtained were conflicting (Okada and Hayashi, 1922; Schmidt, 1929; Mour-Eldin and Wilkinson, 1955).

It was observed in the present study that the serum amino acid pool of blood is significantly depleted in nasopharyngeal carcinoma even though the amino acid nitrogen content of whole blood is increased. It is suggested that the disease might in some way disturb the transport and localization of free amino acids among the different constituents of blood as a result of disruption of the permeability and transport mechanisms of the cells.

It has been shown in the present investigation that the administration of 3-methylcholanthrene to rats results in an increased excretion of ascorbic acid in the urine, and a decrease in the concentration
of ascorbic acid in liver. The concentration of the vitamin in the adrenals and blood was unchanged.

The mechanism by which 3-methylcholanthrene stimulates the synthesis of ascorbic acid is not known. Burns et al. (1960) suggested that the increased ascorbic acid synthesis was due to an adaptive response to the carcinogen, and was the result of increased metabolism of glucose through the glucuronic acid pathway. The increase was abolished by adrenalectomy (Conney et al., 1959a), and not affected by hypophysectomy (Conney et al., 1959a) or nephrectomy (Burns et al., 1960). Studies by Touster and Hollman (1961) on ascorbic acid synthesizing enzymes failed to show any effect of 3-methylcholanthrene on these systems.

The present investigation tends to suggest that the 3-methylcholanthrene influences the ascorbic acid metabolism by either -

i) increasing the rate of glycogenolysis, or

ii) increasing the rate of glucomogenesis from free amino acids of serum.

Further investigation is necessary before any definite conclusions can be drawn.
One of the important and interesting features observed in the present investigation is the capability of ascorbic acid to counteract the effect of the carcinogen, 3-methylcholanthrene and its capacity to minimize the carcinogenic activity. Of course, this observation is neither original nor first, earlier investigators working on the problem have also noted similar findings. However, it is worthwhile to investigate the problem further on the hypothesis; a multiflank attack for solution of the dreaded disease is essential and any suggestion for that, however irrevalent, it may appear in the preliminary stage is to be tried experimentally.