Chapter I

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The suggestion that tumors may arise through extrinsic action first arose from the observation of Pott (1) regarding the high incidence of scrotal cancer among chimney sweeps. The experimental production of cancer in animals is of much more recent origin, dating from the observation of Clunet (2) on the development of sarcomas in rats irradiated with x-rays, and of Yamagiwa and Ichikawa (3) on the carcinogenic action of coal tar on the rabbits' skin. A large number of chemical carcinogens have been discovered since and used in the induction of a variety of tumors, the azo dyes being an important class of the same.

Studies on the carcinogenic effects of amino azo-dyes can be said to have begun with the observation of Fischer (4), on atypical growths in the ears of rabbits following injections of scarlet red, and of Schmidt (5), who succeeded in inducing proliferation of liver cells in mice with this compound. Later, Yoshida (6), and Sasaki and Yoshida (7), demonstrated hyperplastic proliferation of liver cells in rats fed aminoazotoluene. Yet another compound of this group, viz., 4-dimethylaminoazobenzene (DAB) was found to produce hepatoma in rats when incorporated in their diet (8).

These observations led to investigations on the molecular structure of these compounds and the synthesis of more powerful compounds in this series such as 3'-methyl-4-dimethyl-aminoazobenzene (3'-Me-DAB) and several mono and polyfluoro derivatives (9-13). DAB and 3'-Me-DAB in this series have been widely used for the production of primary hepatoma in experimental animals.

Although the carcinogenic effect of DAB and its derivatives could be definitely demonstrated, the suggestion that their action is not absolute but depends on a variety of other factors such as diet arose from the observation that the addition of liver to a rice-carrot diet reduced the incidence of tumors (14). This finding gave rise to systematic studies on the role of individual nutrients such as vitamins, amino acids etc., or combinations of the same, on the induction of experimental cancer.

With regard to vitamins, whereas riboflavin has been reported to retard the onset of tumors in animals treated with DAB, other vitamins such as pyridoxine, B_{12} and biotin would appear to have the effect of accelerating the same (15). No effect could be observed in the case of thiamine, pantothenic acid and niacin (15).

Similar investigations have been made on a few of the amino acids, but they are far from presenting a clear picture and point to the operation of complex variables. For instance, cystine was found to have no effect on tumor production when incorporated in a polished rice-carrot diet but when the basal diet was reinforced with 6 per cent casein, a marked decrease in the incidence of tumor was observed (15). On the other hand, the incorporation of methionine was found to result in a slight decrease in such incidence whereas that of choline was found to have no effect although it was found to reduce gross cirrhosis (15).

Studies on the effect of dietary protein have yielded conflicting results. A few groups of investigators could observe no change in tumor incidence when the level of dietary casein was varied from 10-48% (16-19). On the other hand, an increased incidence on low protein diets is suggested by the observation of Griffin et al. (20), that with 24% dietary casein the incidence is less as compared to 12%, and of Opie (18) that a low protein diet is associated with the formation of cholangiomatous type of tumors.

Similar inconsistent results have been observed with regard to the effect of dietary fat. Diets containing hydrogenated coconut oil have been reported to markedly

retard the onset of tumor by DAB, whereas those containing corn oil or its fatty acids have been found to accelerate the same (20-23). However, it would appear that the difference is not reducible to that between saturated and unsaturated fatty acids from the observation that raising the olive oil content of the diet from 10% to 20% had the effect of reducing the incidence of tumors (19). It has been suggested that the differential effects observed may depend on the amount of riboflavin which can be stored in the liver (24,25).

Studies on the histological changes during carcinogenesis showed that the primary action of DAB is to damage parenchymal liver cells (26) with a concomitant reduction in cytoplasmic nucleic acid followed by focal regeneration of the former around portal spaces. Also, hyaline inclusions in peripheral and central parts of lobules were observed with 3'-Me-DAB and 4'-F-DAB respectively (26). A change to parenchymal-type cells has also been observed in hyperplastic bile duct cells. However, though the origin of tumors induced by DAB has been the subject of much debate, the conclusion seems warranted that tumors induced by DAB or its derivatives have areas of not only hepatoma but also adenocarcinoma and cholangioma and that they are all derived from hepatic cells (26).

Attempts were next made to elucidate the relationship between the carcinogenic action and chemical constitution of these compounds and also their fate in the susceptible animals. Such studies have shown that atleast one N-methyl group is involved in the binding process and that the binding takes place at position 2 or 6 which has therefore to be unsubstituted for carcinogenic activity (27). This apparent requirement for one of the two positions (2 or 6) suggests that the mode of operation of the carcinogen may be through either a re-arrangement of structure involving one of these sites or binding to the proteins of the host tissue or both. The formation of a protein-bound derivative appears to be a necessary step from isotope studies with labeled DAB showing that a portion of the dye combines with certain liver proteins and that this fraction is important in the induction of liver tumors (27,28). However, that this is not a sufficient step is suggested from the observation of normal levels of protein-bound dye in the livers of hypophysectomized rats which are found to be very resistent to tumor induction (29). High levels of bound dye have also been observed in the livers of rats fed 2-methyl-4-dimethylaminobenzene and 3-Me-DAB although these dyes have much less carcinogenic activity than the parent compounds (30).

The type of proteins involved in the formation of the protein-bound derivative has been studied by a number of investigators (31-38). Of interest is the finding that 80% of the protein-bound dye in the supernatant is associated with a group of relatively basic proteins and that tumors which develop subsequent to dye feeding are found to be characterised by low levels of these proteins, designated as 'h' proteins by Sorof and his associates (39). This would appear to suggest that the deletion of specific proteins as a consequence of their interaction with the carcinogen may be crucially involved in the process. This hypothesis receives a certain degree of support from the studies of Weiler (40,41) and of Hughes et al. (42,43).

These observations led to further investigations on the nature of the dye-bound proteins and dye-containing peptides (30,44,45). Recent reports have shown the presence of sulfur in these fractions (30). Interest has also centered around the nature of the binding process and the binding has been suggested to take place at position 2 by Miller et al. (46) and at position 3, by Terayama et al. (47).

The next step was to explore the biochemical differences between normal and neoplastic tissues so that the sites of biochemical lesions responsible for and associated with

neoplasia could be located and possibly exploited. Such approaches led to studies on changes in the levels of carbohydrates, proteins, lipids, nucleic acids and enzymes during neoplastic transformation and possible alterations in metabolic pathways.

Regarding changes in carbohydrate level, a decreased level of glycogen in primary hepatoma as determined by chemical and histochemical methods has been generally reported (48-50). Glycogen depletion and certain histochemical changes have been found to precede bile duct proliferation. These changes could be detected earlier than other changes which became manifest with the development of tumor. That the cessation of glycogen storage commences very early during the development of tumor has also been observed electron microscopically by Porter & Bruni (48). These studies would appear to be consistent with the suggestions of Orr and Price (51), and Graffi and Hebekerl (52) that a lowered glycogen content does not reflect reduced food intake, but, rather, lesions in the metabolic pathway.

With regard to protein, it has been found that the contents of protein nitrogen and RNA are markedly reduced in tumor homogenates, the same being respectively 23% and 36% of those in normal liver (48). DNA, on the other hand,

is reported to be present at high levels in tumors induced by DAB (53-55).

Essentially the same picture has been obtained at the sub-cellular level, protein and RNA in the mitochondrial fraction showing a decrease and DNA in the nuclear fraction showing an increase. The nuclear fraction, however, was found to show an increase in protein content in contrast to the observations made in whole tissue and mitochondrial fraction (56-58). Thus, a differential change in protein content would appear to take place in the nuclear and mitochondrial fractions.

Several studies have been made on the amino acid composition of normal liver and hepatoma (59,60) and their general trend has been to suggest the absence of marked differences between the two. However, glutamine is reported to be almost absent in most of the tumor tissues (61-65). Further, particular protein fractions of liver tumors are reported to show a decrease in methionine and an increase in cystine content as compared to similar fractions from normal liver. Also, it would appear that the homogenates of livers from rats fed carcinogenic azo dyes have a greater resistance to heat flocculation (66).

As regards changes in lipid composition in hepatoma, an increase in both free and esterified cholesterol and a marked decrease in phospholipid content (67-69) have been reported. The latter would appear to be specific to hepatoma since no such change was observed in the case of regenerating liver.

A general decrease in vitamin content is suggested by the observation that in tumors induced by DAB, the levels of thiamine, riboflavin, B_6, biotin, and pantothenic acid range from one-fourth to one-tenth of those in normal liver (70). The levels of associated coenzymes were also found to be greatly reduced (71).

Much interest has naturally centered around the differences in metabolism between normal and neoplastic tissue and several studies have been carried on changes in enzyme patterns with the development of tumors. These studies show a progressive decrease in a number of enzymes such as asparaginase, histidase, esterase, rhodanase, p-amino hippuric acid synthesizing enzyme, catalase, uricase, arginase, xanthine oxidase, β-glucuronidase, transaminases and cysteine desulfurase (72-75). To this group must be added tryptophane pyrrolase, TPNH-cytochrome c reductase and transhydrogenase

which were found to be almost absent in tumor tissue (48,76). Other enzymes, such as guanase, choline oxidase and fatty acid deaminase were found to remain fairly steady in the precancerous stages and to show a sudden decrease with the onset of hepatoma (72). On the contrary, aspartic transcarbamylase, alkaline phosphatase, glutaminase, aminopeptidase, cholinesterase, and the RNA and DNA depolymerases were found to increase progressively, the polymerases, however, returning to normal with the manifest onset of hepatoma (48,74,75,77). A study of 5'-adenylic acid deaminase in a variety of hepatomas showed that the activity was nearly the same in primary hepatoma and normal liver, but showed increased levels in Novikoff and transplanted hepatomas (78).

The decrease in DPN-pyrophosphorylase found in certain tumor tissues (79,80) would appear to be consistent with the observation that they have a lowered DPN content (81) which in turn might perhaps account for the lowering of oxidative phosphorylation and restoration of the same to normal levels with the addition of DPN. Further, mitochondrial preparations of tumor tissue are found to show an increase in the activity of DPNase although no differences are found between the whole tissue homogenates of normal liver and hepatoma (82,83). A

similar increase has also been found with regard to the TPNase activity of the mitochondrial preparation (83).

Interesting observations have been made on enzymes thought to be involved in the metabolism of aminoazo dyes (84,85a). The activity of N-demethylase is found to be markedly diminished in primary hepatoma produced by 3'-Me-DAB (48). The observation that the gross incidence of tumor is reduced by the administration of hydrocarbons such as methylcholanthrene (85b,c) suggests that the same may arrest or retard azo-dye carcinogenesis by enabling the liver to maintain its ability to metabolize the dye.

Among the glycolytic enzymes, phosphorylase and glucose-6-phosphatase were found to be markedly decreased in hepatoma while lactic dehydrogenase was found to show no change (48,86-88). Phosphohexokinase, on the other hand, was found to show a 3-fold increase in activity (89).

Recent studies by Maley and Maley (90) show an increase in deoxycytidylic deaminase in hepatoma and also the appearance in the same of thymidylate synthetase which is found to be almost absent in normal liver though present in varying amounts in other tissues. The former enzyme has been detected in a variety of transplanted and primary

hepatomas. Although this enzyme was earlier reported to be absent in Dunning and Morris hepatomas, as well as in normal liver, more recent work has shown its presence in these tissues in very low concentrations. On the other hand, thymine reductase was found to be present in Morris hepatoma as also in normal liver but absent in other hepatomas (91). This experimental approach has suggested the classification of hepatomas into three groups according to whether deaminase, reductase, or neither, is present (91).

Although comparative studies on enzymes in different hepatomas point to the existence of certain common enzymatic lesions, considerable differences are found between one hepatoma and another. For instance, the lesions are found to be much more extensive in Novikoff hepatoma than in Morris hepatoma, both being of the transplanted type (91-96).

It would thus appear that though several differences in enzyme pattern exist between normal and cancerous tissue, considerable differences have also been found, not only between primary and transplanted tumor, but also between different transplanted tumors so that no specific enzyme pattern generally characteristic of tumor tissue can be said to have been found. The meaning of the reported differences in terms of relevance to malignancy remains therefore obscure.

The studies thus far cited have been generally concerned with alterations in specific enzymes. A different approach to the problem was made by making comparative studies on the operation of different metabolic pathways in normal and neoplastic tissues.

The rapid rate at which tumor tissues are found to grow prompted investigations on possible alterations in oxidative metabolism in these tissues. Several studies have been directed towards measuring the activity of certain segments of the cycle using oxygen consumption and carbon dioxide production (97-102), glycolytic and oxidative enzymes (88,103-108), and co-factors of electron transport system (109,110), or by following the utilization of the various intermediates of Embden-Myerhof scheme and the tricarboxylic acid cycle (89,98,111-114). Although the general trend of these studies has been to suggest an increase in anaerobic and aerobic glycolysis in the tumor tissue with an excessive production of lactic acid (101,115), the extent to which the citric acid cycle operates in these tissues has been the subject of controversy (100,101,116).

The changes in the over-all operation of the glycolytic pathway in rat liver during carcinogenesis were investigated

by Nakatani et al. (117), who found a steady increase in anaerobic glycolysis but no change in aerobic glycolysis. The former observation could not be replicated by Orr and Stickland (118), but has been confirmed by Burk (119) who further reports an increase in aerobic glycolysis as well with gross tumor formation.

As regards the operation of tricarboxylic acid cycle in liver tumor, although Brown et al. (120) found evidence for its operation from their studies using labeled acetate, propionate, octanoate, pyruvate and glucose, the rate of operation would appear to be considerably reduced on the basis of the marked decrease reported in the activities of succinic dehydrogenase and cytochrome oxidase in rat hepatoma (121). This suggestion is supported by the recent report of Dajani et al. (122) who find a reduction in the activity of this cycle in different tumors.

The observed differences in oxidative metabolism led to comparative studies on tumor and normal mitochondria. The observation that tumour tissue has fewer mitochondria (123-125) would appear to be consistent with the decreased activity of some of the respiratory enzymes generally found in this tissue. An abnormal swelling of hepatoma mitochondria has been observed by Emmelot et al. (126,127) who also report

a decrease in the rate of oxidative phosphorylation in the same compared to normal liver mitochondria. The difference between the two tissues, however, was found to disappear on the addition of DPN (110,128). Also, the qualitative similarity of tumor and normal mitochondria, and of the mechanism of oxidative phosphorylation in the same, is apparent from the studies of Aisenberg (129,130).

Studies carried out on lipid metabolism show that the capacity to oxidise fatty acids is retained by tumor tissue (131,132) although the rate of oxidation may be altered. For instance, the oxidation of aceto-acetate is found to be increased in hepatoma but the formation of the same from fatty acids is decreased (133). On the other hand, the capacity to oxidise hexanoic and octanoic acids would appear to be considerably reduced (134). The marked reduction in octanoic oxidase in the liver of 3'-Me-DAB fed animals would appear to be consistent with the latter observation (135).

Similarly, several alterations have been found in phospholipid metabolism. The liver phospholipid level was found to show a progressive decrease during thioacetamide carcinogenesis (48) and a sudden decrease with the manifest onset of hepatoma in DAB-fed animals (48). The latter,

however, were found to show a progressive decrease in the incorporation of labeled phosphorus from the middle of the precancerous period. A depletion of phospholipid content and a diminished turnover rate have also been observed in the case of Novikoff hepatoma (48). These changes appear to be specific to hepatoma since similar decreases have not been found in liver damage.

The ability to synthesize cholesterol from acetate and/or glucose by tumor *in vitro* would appear to be retained by certain primary azo-dye hepatomas although a slight reduction in the same has been suggested from acetate (136). However, no difference in the conversion of labeled acetate to cholesterol in precancerous liver or resulting hepatomas could be detected by Medes *et al.* (137).

Most of the work with regard to protein metabolism has been concerned with the protein and amino acid requirements of tumor tissues. The utilization of plasma proteins for protein synthesis by tumors has been demonstrated (138). However, a comparison of slices from normal and neoplastic livers demonstrated that hepatoma cells can synthesize only 30-50% as much albumin as normal liver slices (48). Further, an increased synthesis of amino acids from glucose is

suggested by the observation that when liver and hepatoma slices are incubated with uniformly labeled glucose, the radioactivity of the proteins in the latter are from five to ten times greater than that in the former (139). This observation has been confirmed by Zamecnik et al. (140) who conclude, however, that the metabolic pathways are essentially similar in normal and neoplastic tissues. On the other hand, the incorporation of labeled leucine in microsomal protein is found to be less in hepatoma than in either normal or regenerating liver and more so when the microsome fraction is supplemented with ATP and phosphoenol-pyruvate (48). These results appear to be of added interest because of the general decrease reported in microsomal and mitochondrial protein in neoplastic tissues.

Yet another interesting observation has been that the incorporation of labeled lysine in liver and plasma proteins is increased in the liver of 3'-Me-DAB fed rats accompanied by a decreased production of CO_2. A marked depression of urea synthesis was also observed (141a,b,c). This led the authors to suggest that the amino acids which show decreased ability to act as precursors of urea during the process of liver tumor formation may be preferentially

shunted to other metabolic processes, such as protein synthesis. Some reinforcement for this suggestion is obtained from the finding that there is greater incorporation of labeled histidine in the liver proteins of dye-fed animals (48).

Studies conducted with regard to nucleic acid metabolism have likewise pointed out certain differences. To elaborate, Werkheiser and Visser (142) found that aminouridine greatly inhibits the incorporation of labeled precursors into both purines and pyrimidines of normal liver but not of hepatoma, thus pointing to the occurrence of alternate pathways in tumors. The studies carried out on the utilization of uracil-2-C^{14} in rat liver and induced hepatomas show its increased utilization for RNA synthesis during carcinogenesis (143,144). Further, azo-dye induced liver tumors were found to incorporate C^{14}-adenine into both DNA and nuclear PNA to a greater extent than normal liver (145). These authors also report that the uptake of P^{32} greatly increases in the livers of rats fed 3'-Me-DAB and show a further increase when tumors become manifest (48).

It would appear from the data thus far available, that the de novo pathway leading to the major nucleotide purines

and nucleic acid purines is qualitatively similar in normal and neoplastic cells and both precursors and pyrimidines are incorporated into the nucleic acids at rates which are of the same order (146). Further, no qualitative differences in pyrimidine nucleotide and polynucleotide synthesis of normal and neoplastic cells have been reported. However, there is some evidence that the capacity of rapidly dividing neoplastic cells to catabolize purine, and purine nucleoside is significantly lower than that of non-dividing tissues such as liver (146).

It will be seen from the foregoing that a number of approaches have been made with a view to identify the biochemical differences between normal and neoplastic tissues. These studies began with experiments on the whole animal and progressed through studies made on isolated organs, tissue slices, homogenates, cell fractions, and finally, purified metabolic factors.

In this as in several other areas of investigation the tissue culture technique is being exploited as a powerful tool in determining the precise nutritional and metabolic characteristics of cells, tissues, and organs, after liberating them from the influence of the organism as a whole. This has been enabled by the spectacular advances

in techniques, including the development of fully defined synthetic media capable of supporting the metabolism and/or growth of such cultures. The development of this technique in its various stages has been reviewed by a number of workers (147-153).

The potentiality of this tool in identifying the differences in the metabolism and nutrition of normal and neoplastic tissue is apparent from the identification of biochemically distinguishing characteristics between different tissues by the use of this technique. To cite a few instances, observations have been made of the non-essentiality of glutamine for chick fibroblasts as compared to its essentiality for rat fibroblasts (154), and the rather unusual requirement of asparagine for Walker Sarcoma 256 (155), glycine, for monkey cells (152), pyruvate, for mouse leukemia 388 (152), and serine, for rabbit fibroblasts (156). These studies have also brought to light the fact that substantial changes take place in metabolism, morphology, physiology and genetic equipment during cultivation in vitro (152)157-159). Of particular interest is the spontaneous development of malignancy in strains derived from normal cells (160) and the reverse phenomenon in those derived from malignant cells (161).

While the identification of metabolic differences between normal and neoplastic tissue has been the aim of several investigators, the choice of the metabolic variables to be investigated has generally had to be made on a more or less 'a priori' basis for want of more suitable guiding criteria. In this context the use of the tissue culture technique may prove most valuable, as a precise determination of the nutritional characteristics of the tissues may provide a pointer to the underlying differences in metabolism and enable a more systematic investigation of the variables involved.

This approach was sought to be taken in the present investigations which were aimed at the identification of the biochemical differences between normal rat liver and dye-induced hepatoma with particular regard to the requirement and metabolism of glucose and aminoacids. Although the growth capacity of adult rat liver is highly diminished when cultivated in vitro (162), studies made in this laboratory showed that explants of liver tissue can be maintained in a metabolically active state during such cultivation so that such an approach was technically feasible. Explants rather than trypsinized cells were chosen because of possible alteration in morphology and

metabolism of cells reported to take place during the process of trypsinization or disorganization (163,164). The differences in nutritional characteristics were followed up by investigations of the presence of corresponding differences in enzyme make-up of the tissues. To ascertain that any differences found are not due to those found generally between fast-growing and normal tissues, comparative data were obtained on newborn and regenerating liver as well. Attempts were also made to partially purify and characterize some of the enzymes of rat liver. The results of these investigations form the material of this thesis.