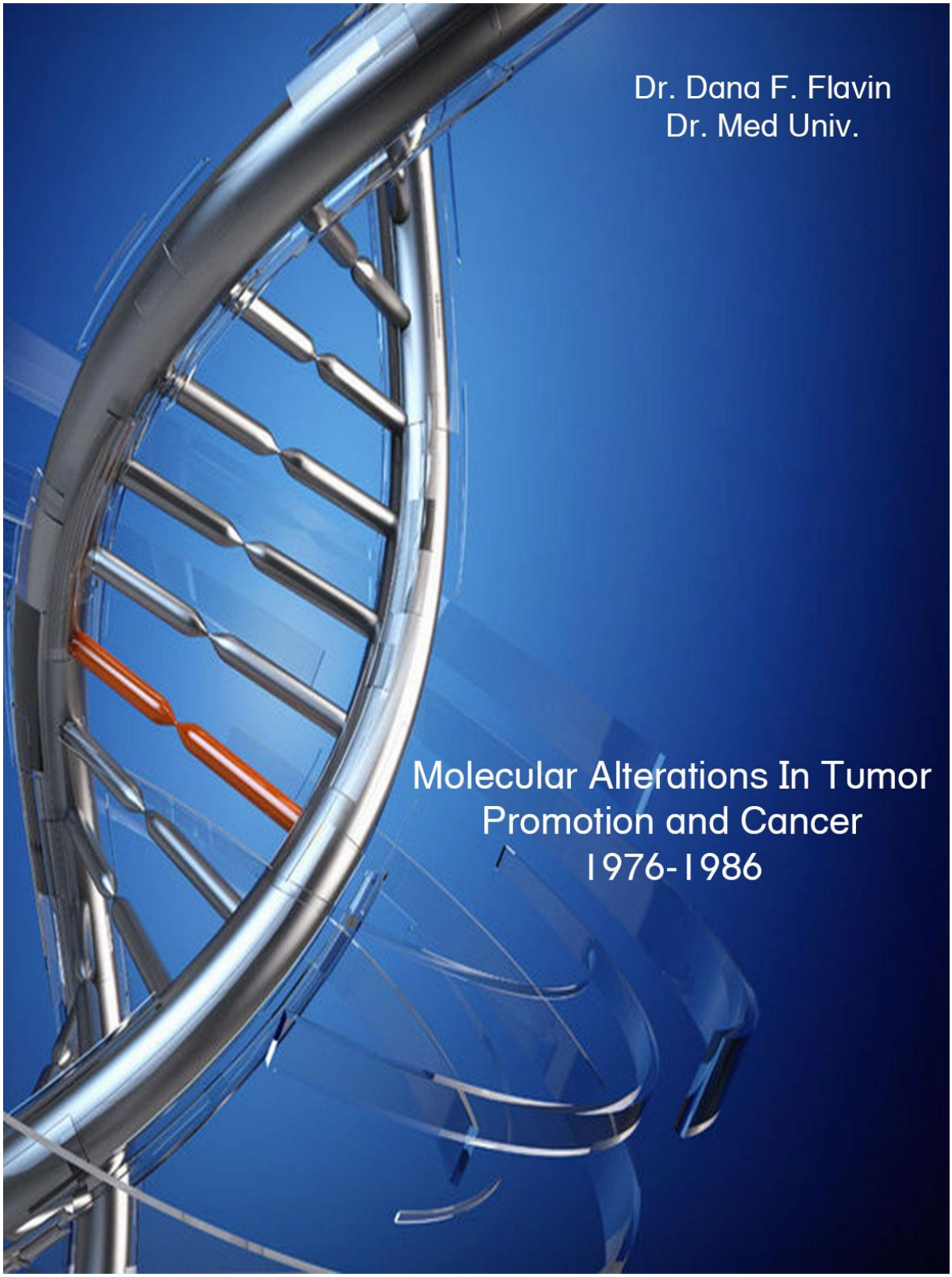
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Molecular Alterations In Tumor
Promotion and Cancer
1976-1986

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ABSTRACT

The basic molecular biology involved in tumor promotion and cancer incorporates two major systems in medicine involving inflammatory processes. The first system which can lead to cancer by chronically damaging cells is also found in viral infections and autoimmune diseases. The second system follows cancer when it has already developed. In this system, after cells have had their DNA irreversibly damaged there is an autonomous replication of cancer cells. The inflammatory processes seen in this latter cancer system actually stimulates cell replication without causing toxicity to the cancer cells. This is different to the inflammatory processes seen in viral and autoimmune diseases, where the inflammation leads to cell destruction. By understanding the different molecular biological parameters in these systems we have the possibility to either prevent tumor promotion in the first system and decrease cancer growth in the second system utilizing the same basic mechanisms of inhibiting the inflammatory parameters.

One of the major biological substances associated with tumor promotion, viruses, autoimmune disease, and cancer are the prostaglandins. Prostaglandins (PG's) are products of arachidonic acid, poly unsaturated fatty acids, that play many roles in diseases. Depending on the disease and the AA cascade, inflammatory or non inflammatory PG's are synthesized. The fatty acid substrate plays a major role in the synthesis of these PGs. A prime example of this is seen using omega-3-fatty acids altering the major substrates for prostaglandin and other cytokins synthesis. Whereas AA contributes to PGE2, an inflammatory cytokine responsible for pain in many diseases, when omega-3- fatty acid intake levels are increased in animals and humans the synthesis of PGE2 dramatically decreases. In tumor promotion and cancer, each molecular biological target can be influenced and stimulate or block cell replication. Direct cellular damage and/or activation at many levels in the cells can lead to cell replication, which is irreversible after a certain point in tumor promotion. By understanding each cellular target and its influences in causing cancer or reversing it, we are able to develop chemical interventions from nutrients and nutrition that can greatly effect the development and prognosis of this disease.

1.0 Membranes

1.1 Surface Active Agents

These agents are tumor promoters for the skin, stomach and colon. Iron dextran, sterilized dimes, concentrated solutions of glucose, other sugars and Na+Cl-induced sarcomas at the injection site (1) (2). Tweens, spans and Na+ oleates are also surface acting agents (3). Even pellets of inert materials (glass, paraffin wax or cholesterol) implanted in the bladder of rats induced tumors (4, 5).

Ethanol is a surface acting agent. The consumption of alcohol is associated with increased incidence of cancer of the mouth, pharynx and also the esophagus (6). Ethanol is capable of stimulating xenobiotic metabolism in the liver and is associated with an increased proliferation of smooth endoplasmic reticulum (7).

In addition to surface acting changes, osmolality changes and irritants to the membrane will enhance tumorigenesis. The urine of rats is mutagenic as is the urine of cigarette smokers (8). This may be deleterious when there is also present a promoter which is capable of producing any of the above changes. The possibility of sodium saccharin acting as a promoter through an osmolality change could be considered in this area.

An enzyme dependent on the intactness of the membrane and on lipids is glucose-6-phosphate, (9). It is lowered in liver cells treated with a promoter. This enzyme is Mg++ dependent and characteristically found in the endoplasmic reticulum of vertebrate liver for the purpose of catalyzing exergonic hydrolytic cleavage of glucose-6-phosphate to leave a free glucose (10).

In rats, bile salts promoted experimental cancer. Choles-tyramine, a bile salt binding agent, promoted colon tumors induced in rats by dimethylhydrazine (11). The bile salts may also be considered in tumor promotion. Higher fecal bile acid levels were found in patients with colon cancer (12, 13).

The bile acid lithocholic acid, a metabolite of cholesterol, promoted colon cancer in rats pretreated with N-raethyl-n-nitro-n-nitrosoguanidine (14). When rats were fed a cholesterol free diet there were fewer tumors which appeared at a much later date than did those of rats fed the same diet with cholesterol added (15).

In conditions favoring conversion of bile acids to more active substances the incidence of biliary tract cancer and of several other G.I. tumors is increased. Finally, ulcerative colitis increases the risk of bile cancer by a factor of ten (16).

1.2 Saturated and Unsaturated Fatty Acids and Tumors

A diet high in fats increases the incidence of chemically induced skin tumors as well as spontaneous mammary carcinomas and hepatomas in the mouse (17). Unsaturated fatty acids appear to have a greater effect of tumor incidences in rats and mice. In rats fed unsaturated fatty acids along with a carcinogenic azo dye an increase in tumors was demonstrated (18) (19). Mice fed a high triene (soybean oil, safflower oil, oleic acid) diet had a higher incidence of liver tumors than corresponding animals fed a high saturated fatty acid (palm oil, palm stearated oil) diet (20). Twort and Twort found that oleic acid stimulated tumor formation when applied either concurrently with a carcinogenic hydrocarbon or continuously following several

applications of the hydrocarbon (21). The more frequently oleic acid was used the steeper was the incidence of tumors (22). Methyl esterification of oleic acid increased its activity as a tumor promoter (23). Lauric acid also exhibited distinct tumor promoting properties. The strongest tumor promoters known so far are the diterpene esters (24) which are extremely lipophilic (25).

In a diet deficient in polyunsaturated fatty acids there was an inhibitory effect of breast tumors in ADK-1 mice. This inhibition appeared to be strain specific in that only BALB/c mice but not DBA/2 showed this inhibition, and involved the host immune system (26) Recent studies have shown that saturated fats enhance tumor yields as effectively as unsaturated fats provided they are fed with a small amount of a polyunsaturated fat (27).

Studies of factors that modify the processes of carcinogenesis in experimental animals are important because such modifying factors may alter the outcome of an experiment. These factors may include the species and genetics of the test animals, their age, sex, nutrition and immunological state, as well as factors associated with the test compound such as the vehicle used to suspend or dissolve the compound, the purity of the compound, and its metabolic fate (28).

1.3 Membrane Surface Charge

The tumor promoting phorbol esters are capable of enhancing the phenotypic expression of spontaneous and viral induced loss of contact inhibition (29, 30). Krieg et. al. demonstrated that chalone responsiveness is very low in transplantable epidermal carcinoma (31). There is a change in the electrical charges on the surface of membranes in tumor formation (32) that may be related to the alteration in chalone responsiveness. It is known that metabolic changes of simple sugars as in glycoproteins and glycolipids can cause a remarkable change in cell contact behavior which would be a membrane mediated process.

Malignant cells have generally been reported to have a higher negative surface potential than normal cells, demonstrated by cell electrophoresis and histochemical staining, and it is known that the most important group contributing to the surface charge of the cells is the carboxy1 group of sialic acid (N-acetyl-neuraminic acid) (Choriogonodotropin - CG). Because several of the long carbohydrate chains of CG have an open sialic acid terminal group, any increase of CG content in the plasma membrane will increase the negative potential. This physical phenomenon may explain the “immunosuppressive” activity of the hormone as a simple “repulsion” effect, providing a possible mechanism of action for the immunological inertness of CG as well as an explanation of the effects of neuraminidase in the cancer cell.

The presence of CG in the malignant cell is of fundamental importance due to some of the biological properties of this protein. It is a potent stimulator of the de novo synthesis of cholesterol, one of the main constituents of the cell membrane, and possibly protects against immunological attack.

It is very possible that with the constant rapid movement seen in tumor cells, (33) the surface antigens that are exposed are incomplete. If this is the case antibodies to many tumor associated antigens could be insufficient to destroy the tumor, that is they not only would be parital antibodies but the chance of the specific antigen surfacing would be also be lessened by movement.

In addition to the constant fluctuations in microvillous structures for a chameleon-like quality, fluctuation in antigens are also possible.

Evidence of other "placental proteins" by nontrophoblastic neoplasm have been shown in addition to a choriogonadotropin-like membrane associated antigen in cancer cells (34). Sellen and his associates detected free alpha CG-like material in R-3230 AC rat mammary adenocarcinoma. These investigations also demonstrate the presence of significant levels of CG in the tumor extracts by bioassay in vitro, indicating the presence of the complete hormone (35). Tumor cell surfaces are enormously complex with constant modulation, redistribution loss of cell surface components aggregation, localized nonspecific or specific coupling or agglutination with nonmembranamous substance to prevent complement mediated immune lysis as Nicolson and Poste termed "immunoevasive properties," - (36). (See diagram 2)

1.4 Glycolipids

Glycolipids and especially gangliosides (sialogly-cosphingolipids) are enriched at the surface of the cell and function as specific membrane receptors. The hydrophobic portion of the ceramide moriety of the ganglioside is anchored in the membrane and consists of a long chain fatty acid linked through an amide bond to sphingosine. Sialic acid levels, elevated in fetal liver and at birth, remain more or less constant in the adult. In poorly differentiated, well circumscribed hepatomas however, maximum elevations of sialic acid are attained. In neoplasia from chemicals, viruses and tumors of spontaneous growth, a depletion of complex gangliosides is seen and an accumulation of simpler precursor gangliosides has been related to an increase in bio-synthetic enzymes giving rise to the latter. These simplications of glycolipid patterns emerge as late events in tumorigenesis (37) cultivated human epidermal deratinocytes that stop growing form an insoluble envelope under the plasma membrane that consists of protein cross linked by epsilom-(gamma glutamyl lysine) that depends on Ca++ for activating the cross-linking translutaminase (38). In addition to the influence of Ca++ on membrane stability, Ca++ is necessary in the activity of the Ca++, Na+, and K+ pump-ATPhase. With the presence of Ca++ two Na+s are removed from the cell and on K+ enters. This maintains a more negative charge inside the cell. Ca++ combined with calmodulin aids in the removal of free Ca++ from the cell (39). It is thought that Vitamin A maybe assisting the enzymes necessary for reestablishling the more complex ganglioside formation carrying mannosyl residues across the lipid bilayer (40).

Vitamin A may be working partially through Ca++ to exert its effects, perhaps by keeping it in the membrane to act with essential enzymes. Vitamin D causes the induction of alkaline phosphatase which may act as a calcium binding protein. In tumor cells the amount alkaline phosphatase and Ca++ are both decreased. Local application of Vitamin D2 and D3 caused inhibition of tumor formation in hamster cheek pouch induced by 9, 10 Dimethyl-l,2-benzanthracene (41)*.

Gangliosides, especially GM2, are red blood cell membrane sites for the phorbol esters with tumor promoting properties. Inhibition of phorbol ester hemolysis of RBC was obtained with the addition of gangliosides GM2 being the most active inhibitor. The glycoprotein fetuin also inhibits hemolysis but platelet aggregation by phorbol esters was not inhibited by gangliosides (42).

1.5 Receptors

There is a shift from a B-receptor response to an alpha receptor response in cells treated with the phorbol ester TPA (43). The suppression of B-adrenergic stimulation by TPA is considered a promoter induced and promoter specific derangement of epidermal structure rather than a direct interaction of the phorbol ester with the receptor: The activity of epidermal membrane bound adenylcyclase, however, is not influenced by TPA (44).

The phorbol ester TPA suppresses B-adrenergic stimulation after a lag phase of 1 to 2 hours (45). It abolishes the ability of mouse epidermis to respond to B-adrenergic stimulation by an elevation of endogenous cAMP in vivo. TPA causes an increase in phosphodiesterase synthesis (also seen with non-promoter depilatory cream).

Lee and Weinstein have reported that TPA and other phorbol and non-phorbol promoters inhibit the binding of Epidermal Growth Factor (EGF) to EGF receptors by a mechanism that involves neither competition nor decreased affinity. Cells transformed by RNA tumor viruses also lost the ability to bind EGF cell surface receptors (46, 47). Zurhausen et. al. have reported that TPA induces Epstein-Barr virus (48) and activates latent viral genomes (49).

2.0 Prostaglandins

The prostaglandins are among the most prevalent of autocoids and have been detected in almost every tissue and body fluid. The synthesis and release of prostaglandins is affected by nutritional, physical and chemical factors. Their production increases in response to diverse stimuli producing a remarkably broad spectrum of affects. In some situations prostaglandins appear to act as mediators for external stimuli, whereas in other cases they may actually act as hormones on other cells.

The different prostaglandins fall into several main classes -- E, F, A, B, C and D — distinguished by the constituents of the cyclopentane ring. Prostaglandins of the E and F series are the most abundant (1).

Prostaglandins have been associated with tumor promotion, inhibition and cocarcinogenesis. They may also assist in a cascade of events leading toward cellular tranformation.

2.1 Synthesis

In vivo most cells seem capable of synthesizing prostaglandins from essential fatty acid precursors in a stepwise manner, by prostaglandin synthetase (2). The oxidative closure of a cyclopentane or cyclopentene ring in the middle of a polyunsaturated fatty acid chain forms a cyclic peroxide derivative. This endoperoxide is then either isoraerized to yield PGE or reduced to PGF (3, 4). The majority of arachidonate, a precursor of prostaglandins, is found in mammals covalently bound and in an esterified form as a significant proportion of the fatty acids in the phospholipids and in esterified cholesterol. In the esterified form, arachidonate is exchanged between various pools by an acylhydrolase, phospholipase A^2(5). Activation of phospholipase A has been suggested as a common rate limiting step in the hormonal and neurohormonal stimulation (6).

An increase in prostaglandin synthesis and release of arachidonic acid from phospholipids is seen with several hormones including ACTH, TSH, prolactin and epidermal growth factor (EGF) (7).

It is generally believed that endogenous biosynthesis in the absence of added precursor acid depends on the phospholipase-catalyzed release of precursor acids from cellular phospholipid stores (8, see diagram).

2.2 Tumor Promotion and Inhibition with Prostaglandins

Prostaglandins may affect tumorigenicity in many ways. They are synthesized in the cell membrane in normal cells and in various tumors their levels are elevated (9, 10). Smythies et. al. has calculated that the phorbol diester tumor promoter, 12-0-tetradecanoyl phorbol-13-acetate (TPA) may fit the hypothetical receptor of prostaglandins which have been repeatedly claimed to effect epidermal differentiation and proliferation. TPA causes release of arachidonic acid from membrane phospholipids which is associated with a stimulation of prostaglandin synthesis- This

response occurs rapidly, is not blocked by Actinomycin D, is not seen with non-tumor promoting diterpenes and is inhibited by trans-retinoic acid (11) (12).

Kreibich et. al. found that stimulation of phospholipid metabolism was specific for tumor promotion since only the tumor promoting phorbol esters stimulated the choline incorporation into Hela cells (13). TPA treatment of mouse skin was followed by a rapid increase in the rate of incorporation of inorganic phosphate into each different skin phospholipid. The stimulation correlated with the tumor reponse with respect to dose and culture (14, 15, 16).

Prostaglandin E (PGE) synthesis is thought by some to be an obligatory event for the activation of ornithine decarboxylase, stimulation of epidermal cell proliferation and hyperplasia by phorbol esters, ionophore A23187 and cell wounding (17).

TPA released PGE2 very rapidly from cellular membranes (18). In low doses TPA stimulated PGE2 release from macrophages by a series of tigliane, ingenane and daphnane type diterpene derivatives, with a good correlation established between irritant and promoting activity in the mouse skin. The effect was inhibited by indomethacin (19).

In cultured dog kidney cells (MDCK), TPA stimulates the deacylation of cellular lipids and arachidonic acid metabolism. MDCK cells are also stimulated to produce prostaglandins, with an increase in phospholipase activity, by means of metabolically activated carcinogenic aromatic hydrocarbons and aflatoxin B1 (20).

Certain cancers associated with diarrhea such as medullary carcinoma of the thyroid have been shown to raise levels of PGE2 and PGF2 alpha by as much as 20 times. In Kaposis sarcoma, symptoms of pricking pain, itching, edema and sweating are also related to plasma levels of PGE2 and PGF2 alpha. The level of gastric acid is reduced when PG's are given (21).

PGE is thought to serve as an intermediate messenger between the cell surface hormone receptor and the membrane-bound adenylcyclase in some target cells.

2.3 Prostaglandins and Cyclic Nucleotides

According to Ramwell, prostaglandins seem to reduce the level of cAMP by diminishing the sensitivity of adenylcyclase to different hormones (22). Their regulation of the inflammatory response is through this effect on cellular levels of cyclic nucleotides.

cAMP and cGMP appear to control the release of mediators of inflammation. The initial formation of endoperoxides from arachidonic acid has been shown to stimulate cGMP, which ties with the inflammatory effects of prostaglandins as they are released. Later, however, when specific prostaglandins such as PGE1 accumulate at the site of injury, they seem to be able to raise the level of cAMP in white blood cells and dampen the inflammatory response.

In large quantities PGE1 and PGE2 increase levels of cAMP and thereby reduce extrusion of lysosomal enzymes from leukocytes, prevent release of histamine and slow reacting substance of anaphylaxis and prevent lymphocyte mediated cytotoxicity. PGE2 is elevated 10 times more in rheumatoid synovia than normal synovias, it also stimulates bone resorption and collagen synthesis. PGE2 had inhibitory effects on cAMP at 1 and 5 mg (23). The inhibition of cAMP accumulation is associated with the inhibitory effects of prostaglandins on lipolysis. PGE2 / which has an acidic lipid nature, activates platelet aggregation. The inhibition by

indomethacin of the TPA induced raitogenic response can be overcome by PGE2 but not PGF2. The inhibition of the promoting activity of TPA by indomethacin can be overcome by simultaneous application of *PGF2*, but not PGE2, however neither PG's are promoters (24, 25). PG's E and F are stable in the blood but they are rapidly degraded and inactivated by tissue bound enzymes, some 80 to 90% or more is destroyed by the liver and lungs during a single passage (26). PGE1 plays a major role in the regulation of thymus development and T-lymphocyte function as well as mucin secretion in the intestine (27).

Many cancer cells and virally infected cells are unable to make PGE1 because they cannot convert linolenic acid to gamma-linolenic acid. The production of PGEi is dependent on nutritional factors with linolenic acid, gamma linolenic acid, pyrodoxine, zinc and Vitamin C playing key roles. Inadequate intake of any one of these will lead to inadequate PGE1_ formation and defective T-lymphocytes, thus decreased immunity from cancer (28).

Another prostaglandin that affects cell growth is PGA1_, which profoundly inhibited the replication of both DMSO-treated and undifferentiated friend erythroleukemia cells (FLC). PGA1_ alone induces differentiation in FLC and stimulated hemoglobin production over a five day period (29).

2.4 Prostaglandins as Cocarcinogens

In addition to the promoting and inhibiting effects of prostaglandins some are thought to act as cocarcinogens. It was found that concomitant administration of PGE2 and PGF2 alpha with a carcinogen, 3 methyl cholanthrene (MCA) to mice for two months markedly enhanced the occurrence of squamous cell carcinomas. Only epidermal cell hyperplasia occurred in mice treated with MCA alone. PGE2 and PGF2 alpha can act as cocarcinogens (30), although this may be a promoting effect.

It has been suggested that the oxidation of benzo[a]pyrene (BP) to electrophilic metabolites is dependent on arachidonic acid. The activation of 7,8-dihydro-7,8-dihydroxy benzo[a]pyrene is markedly reduced by the omission of arachidonic acid and is inhibited by the PG synthetase inhibitor indomethacin. 2,3-dimercaptopropanol, butylated hydroxy anisole. Vitamin E, diethyldithiocarbaroate, epinephrine, lipoic acid, and Vitamin C are also inhibitors (31).

2.5 Inhibition of Prostaglandins

Prostaglandin synthesis is inhibited in Essential Fatty Acid deficient animals (32). Mice with diets low in polyunsaturated fats showed a decrease in spontaneous tumors (33). In addition, analogs of the natural fatty acid precursors serve as competitive inhibitors of prostaglandin production (34).

Inhibition of prostaglandins can inhibit TPA induced tumor promotion (35). Aspirin like drugs interfere with the biosynthesis of prostaglandins at the cyclooxygenase stage so that the generation of prostaglandin endoperoxides, PGG2* PGH2, thromboxane A2 and prostacycline are also prevented. Steroids on the other hand, affect prostaglandin synthesis by limiting the availability of arachidonic acid which has at least two main metabolic pathways, only one of which is inhibited by

aspirin (36). Currently only hydroxy corticosteroids, other steroidal antiinflammatory drugs and local anesthetic agents are known to inhibit arachidonic acid release (37). Chloroquine, quinine, quinidine procaine, clomipramine, theophylline and caffeine have been shown to be strong prostaglandin antagonists and weak agonists (38).

Cycloheximide, an inhibitor or protein and RNA biosynthesis, inhibits the phorbol diesters stimulated prostaglandin biosynthesis, the changes in morphology and the increased lipid deacylation but hydrocortisone does not (39).

Indomethacin inhibits tumor promotion by TPA (40). It blocks the arachidonic acid to endoperoxide step and possibly inhibits phospholipase (41). Calcium also affects phospholipase and is required in the binding of PMA (42). Indomethacin irreversibly inactivates cyclooxygenase. At very high doses it can inhibit phosphodiesterase (43). Treatment with indomethacin before application of TPA inhibits the accumulation of prostaglandins, the induction of ornithine decarboxylase activity, and the formation of skin papillomas (44). Hydrocortisone, fluocinolone and other hormone inhibitors of promotion also inhibit prostaglandin synthesis in the skin (45).

It was suggested that prostaglandins may play a role in ornithine decarboxylase induction as well as skin tumor promotion. Prostaglandins may be necessary but not sufficient for ornithine decarboxylase induction to TPA (46) especially with the evidence that dexamethasone will inhibit prostaglandin release without inhibiting ornithine decarboxylase. Dexamethasone, an active inhibitor of promotion, elaborates a protease inhibitor (47, 48, 49), as well as stimulates the production of a phospholipase A2 inhibitor (50).

The changes in prostaglandin synthesis by TPA precede the protease, plasminogen activator by several hours (51) but indomethacin does not effect induction of plasminogen activators. This seems to indicate that the leukotrienes activate plasminogen activator, rather than the prostaglandins.

Evidence has been presented for prostaglandin mediated hypercalcemia and bone resorption in malignancies of both experimental animals and man. Prostaglandins of the E Series have been considered in the pathomechanism of hypercalcemia and osteoclastic osteolysis in cancer patients (52).

2.6 Prostaglandins and Calcium

Calcium was shown to be required in the PMA stimulated DNA synthesis of blocked 3T3 cells, the proliferation of thymic lymphoblasts, and an increase in the secretion of plasminogen activators (53) as well as in the aggregation of platelets caused by exposure to PMA. Even the inital binding of PMA to plasma membranes is modulated by calcium (54) (55) (56).

Calcium is required to stabilize the membrane from excessive Na+ influxes (57). The chloroquine, quinine, procaine (a local anesthetic), group of drugs are able to stabilize membranes, reduce deformability and ion permeability and combine with nucleic acids. Chloriquine can bind to nucleic acids, block unwinding of DNA, inhibit DNA and RNA polymerases, prevent DNA repair and arachidonic acid release (58). Prostaglandins can block the reaction between chloroquine and DNA.

Local anesthetics and calcium compete for the same membrane phospholipid binding site, each being partial agonist/antagonist of each other (59). EDTA, the calcium chelating agent combined with trypsin (neither alone) caused a decrease in cell associated phorbol ester TPA (60).

It has been suggested that prostaglandins and calcium ions probably interact in the regulation of transmitter release and uptake in nerves (61) as well as other cells. In nerve cells there is a low resting membrane potential that is altered by an influx of Na+ and an efflux of K* responsible for an increase in intracellular metabolic activities (62). The changes in membrane associated enzyme function from the phorbol esters (63) are associated with changes in ion fluxes and a concomitant decrease of Na+ K+, Mg++ Ca++ ATPase activity. This follows with an increase in Na+ intracellulariy (64) (65). Vitamin A'appears to reactivate the Na+ K+ pump (66).

Reese and Friedman found that a decrease in ionic Ca++ caused an increase in cell division of bladder culture cells (67). However/ the opposite effect was found in liver cells. Extracellular calcium deprivation arrested the proliferative development of rat liver cells. The addition of calcium or a tumor promoter such as 12, 13-didecanoate, phenobarbital, saccharin or TPA initiated DNA synthesis from these arrested cells (68). Other reports have also pointed to an involvement of calcium in the biological effects of PMA (69). The calcium deprivation in vitro and in vivo reversibly stops proliferative development at that part of the G 1-2 phase in which the cellular c-AMP content transiently rises and the synthesis of the four deoxyribonucleotides begins. Retinols also inhibit at the G 2 phase (70). The evidence points to calcium and the c-AMP surge being co-generators of the signal committing the cell to DNA synthesis (71).

Calcium is essential for prostaglandin synthesis. It is required by Phospholipase A2. The key residues in the mechanism of action of this enzyme are asp and his between which a calcium ion is chelated (72). Calcium is also important in the binding of sialic acid in the membrane which helps maintain its surface charge and contact inhibition. (See 1.4)

TPA facilitated the translocation of calcium into a hydrophobic domain in an artificial system. It is suggested that TPA interferes with ionophore-mediated calcium transport across biological membranes (73).

It is possible that part of the inhibitory action of Retinoids on tumors is through its effect on calcium. Retinoic acid and indomethacin inhibit phospholipase A (prostaglandin synthesis) and ornithine decarboxylase (ODC) (74) (75) . The link between indomethacin and retinoic acid appears to be calcium since indomethacin antagonizes calcium and ODC requires calcium along with cAHP for its induction. Dexamethasone inhibits prostaglandins through the synthesis of a phospholipase inhibitor rather than effecting calcium. It does not inhibit ODC (76) (77).

2.7 Calcium and Calmodulin

Calcium in cooperation with calmodulin, that is membrane bound, the calcium binding protein, can alter c-AMP concentration (78) (79).

Calmodulin that is membrane bound has been reported to be disassociated by c-AMP dependent phosphorylation of membrane preparation (80).

Many enzymes regulated by calmodulin calcium or by calmodulin calcium dependent kinases are also substrated for cAMP dependent kinases as well. These two major second messenger systems are functionally coupled and integrated at the level of protein phosphorylation (81). The calcium dependent form of cyclic nucleotide phosphodiesterase is activated by calmodulin. CAMP increases the affinity of phosphodiesterase for calmodulin. Under physiological conditions the calcium

threshold for phosphodiesterase activation will depend on the amount of free calmodulin (82).

It is thought that adenylcyclase may be turned on by lower concentrations of the calcium calmodulin complex than those that activate the phosphodiesterase. This could cause a transient increase in c-AHP (83). TPA causes a transient increase in-c-AHP (84). Phospholipids also activate phosphodiesterase and simultaneously prevent calmodulin stimulation, suggesting both activation and inhibition of adenylcyclase by the calmodulin calcium complex (85).

Transformed rat kidney cell lines showed a pronounced increase in calmodulin over normal rat kidney cell counterparts. Increased calmodulin have also been observed in virally transformed chick embryo fibroblasts (86).

Some preparations of guanylate cyclase are also stimulated by calcium. The guanine nucleotides lower the Km value of adenylate cyclase for calmodulin and calmodulin is required for activation of these effectors (87).

The possibility has been raised that interconversion of the molecular forms of phosphodiesterase is effected by limited proteolysis or by a concentration-dependent mechanism. There was also reported an irreversible calcium independent activation of phosphodiesterase by limited proteolysis (88).

3.0 Ornithine Decarboxylase

An important enzyme altered in tumor cells is ornithine decarboxylase, (ODC) (1). Its importance comes from the fact that it is the rate limiting step in the production of the polyamines which control the synthesis of the nucleic acids and proteins involved in cell proliferation and differentiation (2, 3, 4). Transformed cells have an increase in polyamine levels (5, 6, 7, 8).

3.1 Polyamines and DNA

One cellular site of action of the naturally occurring polyaraines putrescine, spermine and spermidine is DNA synthesis (9). In chromatin, all cations compete for the binding sites on DNA with histones or protamines, in sufficiently large concentration, cations can displace these proteins (10).

DNA usually exists as a double helix in an extended coil with secondary phosphate groups constituting the bridges between adjacent mononucleotides by linking the sugar of each nucleotide (11). The phosphate groups strongly bind divalent cations Mg++ and Ca++ (12). Local changes in these divalent cations may profoundly affect the chromosomal architecture (13).

The addition of the divalent cations Mg++ and putrescine to a solution of DNA causes an alteration of the DNA from its extended to a condensed form. The trivalent and tetravalent cations spermidine or spermine can also cause condensation of DNA (14).

The acidic nature of DNA indicates that multivalent cations will bind in near stoichometic proportions to the phosphate backbone of this molecule. The polyamines are more active on a molar basis in neutralizing the electrostatic repulsion of the phosphate groups (15) partially hepatectomized rats show an increase in putrescine during the first ten hours (16). However, it appears that a prior accumulation of the polyamine spermidine is required for normal DNA replication and cell division after a partial hepatectomy (17) Regardless of the possible effects of polyamines on DNA synthesis, the time course of ODC activity appears to be independent of the onset of DNA replication in regenerating livers (18).

3.2 Stimulation of Ornithine Decarboxylase

Calcium, asparagine and c-AMP are required for ODC activation (19) however, many other factors will affect the activation and elevation of ODC. These include such things as diet, physical irritation, ultraviolet light and wounding. Hair plucking stimulates ODC activities in rat epidermis and dermis. This is affected by diet. The activities of ODC in plucked and unplucked skin are higher in animals fed a higher protein content in their diet (20, 21). Wounding also induces ODC and promotes carcinogenesis whereas skin massage does neither (22, 23, 24), indicating that induction of ODC follows only those stimuli that are strong enough to irritate or damage.

Serum, serotonin and thrombin can also produce a rise in ODC activity (25). In all cell types, fresh serum containing medium induced ODC and stimulated DNA synthesis (26). Induction in the presence or absence of serum requires both DNA and protein synthesis (27).

In the ultraviolet light induced ODC in the epidermis of hairless mice, the activity preceeded the delayed increase in DNA synthesis (28).

3.3 Ornithine Decarboxylase in Tumors

The half life of ODC is greatly extended in tumors (Papillomas) compared to normal tissue. In a spectrum of Morris hepatomas of various growth rates, ODC activity was preferentially retained or increased whereas other pathways of ornithine utilization, as well as many other enzymes were decreased (29). Cells from three ascites cancers and one fibrosarcoma, on the other hand, showed declining ODC production with progressive growth (30)

ODC activity is not induced by initiating doses of carcinogenic hydrocarbons, however, the completely carcinogenic doses were able to induce the enzyme. S-adenosylmethionine decarboxylase (SAM) activity was also induced after the higher dose (31). At low initiatory doses, benzo(a)pyrene, methylcholanthrene and dimethylbenzanthracene did not induce ODC or SAM, but at higher levels that initiated and promoted, induction was seen (32). This seems to indicate that for induction of tumors the activation of ODC is important in the initial stages only, but that its continous activity is not necessarily imperative.

3.4 Tumor Promoters and ODC

Tumor promoters inhibit terminal differentiation of cells in culture (33). Non promoting hyperplasiagenic agents induced SAM without inducing ODC (34, 35). Every promoter, however, that was tested in mouse skin induced ODC, but the non promoters did not (36, 37, 38).

A single typical application of the phorbol diester tumor promoters (TPA) cause a rapid transient increase in mouse epidermal ODC activity. The stimulation of enzyme activity is dependent on the dose of TPA applied and correlates well with the promoting ability of these doses on mouse skin (39, 40, 41, 42, 43, 44, 45). Two diterpene esters, TPA and the antileukemic agent mezerein are equipotent on a molar basis in inducing hyperplasia inflammation and ODC activity as well as reducing cAMP accumulation in response to beta adrenergic stimulation (46). Its effect on ODC and prostaglandins may be partially through calcium to exert its inhibitory action.

Species specificity is one factor that can influence the affect of chemicals etc. on cancer promotion and induction. A prime example is that the promoting action by croton oil (and supposedly by TPA itself) is species specific for mouse skin and does not operate in rabbit, rat or guinea pig skin (47).

Other compounds may also act as prostaglandin agonists and antagonists including chlroquine, quinine, procaine, quinidine, tricyclic antidepressants and methylxanthines. (48)

Theophylline, a phosphodiesterase inhibitor, that elevates cAMP, inhibited ODC activity in control and gonadotropin stimulated granulosa cells from porcine ovarian follicles (49).

Another study inhibiting ODC through the elevation of cAMP was done on lymphoma cells. Incubation of S-49 lymphoma cells with N 6,02-dibutyryl cyclic AMP (BT2cAMP) decreases the activities of ODC and SAM. Studies in mutant S 49 clones that have altered protein kinase indicate that cAMP dependent protein kinase mediates the decrease in ODC and SAM activities. The dose response pattern for the cAMP stimulated decrease in enzyme activities parallels the pattern for the cAMP stimulated cell cycle specific (G1) growth arrest of s 49 cells (50).

It is suggested that the passage of cells through the cell cycle is required for the maintaining activities of ODC and SAM (51, 52). The retinals inhibit not only ODC induction but also the G1 progression in Chinese hamster ovary cells (53), indicating a cell cycle specific locus for Vitamin A inhibition of growth (54).

Vitamin A is essential for controlling the normal differentiation of many epidermal tissues. Those tissues most prone to cancer (skin, breast, lung, colon, rectum, pancreas, prostate and bladder) require Vitamin A for normal differentiation. In the trachea and bronchi squamous metaplasia occurs in vitamin A deficiency (55)• The morphological alteration in epithelium caused by incompetency of retinoids in the diet are similar to those obtained after treatment with chemical carcinogens (56). The interaction of zinc should be mentioned at this point: Zinc and copper appear to compete, perhaps for uptake, in that there are inverse relationships between serum copper and zinc. Plasma levels of both vitamin A and retinol-binding protein are low in zinc deficient animals, despite the fact that liver stores of Vitamin A are adequate, thus zinc appears to be necessary for Vitamin A metabolism (57).

Ballog demonstrated that epidermal papillomas and carcinomas initiated dimethylbenzanthracene and promoted by TPA can be inhibited by oral administration of retinoic acid during the promotion stage. Not only can retinoids prevent the induction of premalignant lesions in the epidermis, but they can reverse premalignant lesions of epidermis and prostate after they are already formed (58).

Retinoic acid applied concurrently with TPA inhibited tumor promotion in a dose dependent manner, and dramatically inhibited the TPA induced mouse epidermal ODC activity. The degree of inhibition was dependent on the dose and time of application (59).

The most active antitumor synthetic retinoids are the cyclopentyl and trimethylmethoxyphenolethyl ester derivatives of retinoic acid. Their anti-tumoral affects appear to involve a tight binding with specific protein and inhibition of ODC in tumors (60).

Although Vitamin A palmitate can be toxic, the acid form is not stored in the liver and therefore higher levels can be attained (61).

In some cases metabolic activation appears to be required for the retinoic acid's anti-tumor action. This was suggested for mouse lymphocytes treated with TPA. It should be noted that the inhibition of ODC is not imperative for tumor inhibition to occur. Inhibition of both prostaglandins and protease by dexamethasone will block tumor promotion without inhibiting ODC. The inhibition of one essential element in a tumor does not seem to be sufficient for inhibiting promotion or carcinogenesis, without also inhibiting another element. The blockage of one pathway often is compensated for by the activation of another. Along this line Vitamin A will inhibit prostaglandins, ODC and promotion in epithelial tissue, however, it will elaborate the activation of a protease in mesenchynal tissue. For this reason, combinations of

inhibitors such as retinoic acid with dexamethasone are often more effective in non epithelial tissue.

4.0 Cyclic Nucleotides

Two regulatory nucleotides may be implicated in the regulation of gene expression during the cell cycle, and in transformed cells. These nucleotides are adenosine 3',5' cyclic monophosphate (cAMP) and guanosine 3' ,5' cyclic monophosphate (cGMP).

cAMP arises in eukaryotic cells from adenosine tri-phosphate (ATP) by the action of an enzyme located in the cell membrane, adenylcyclase (1). Guanylcyclase, the enzyme activating cGMP is also membrane bound. They often undergo inverse changes in concentration in cellular events in which opposing or bidirectional processes are regulated suggesting a reciprocal relationship between the two nucleotides (2). It has been proposed that the cGMP is the intracellular messenger that induces cell division and limits differentiation and that cAMP inhibits cell division and promotes differentiation, this is the yin-yang hypothesis (3). The increase in cGMP appears to be a result of an increased influx of CA++ (4) which activates guanyl cyclase through an indirect mechanism (5).

Mitogens trigger many quiescent cells into division increasing cGMP to levels approaching cAMP. There is either no change in cAMP (6) or an actual decrease in the concentration of cAMP with a decrease in adenyl cyclase activity (7). cGMP levels as well as guanyl cyclase activity appear to vary in the opposite direction in relation to cell division rate (8).

Mitogens like trypsin and the lectin concanavalin A (Con A) inhibit adenylcyclase which could thus cause a decrease in cAMP.

4.1 Regulation of Cyclic Nucleotides

Regulation of the branching pathways to AMP and GMP is achieved in two ways. The reaction pathway leading from xanthylic acid to GMP requires ATP as a reactant, the pathway leading from inosinic acid to AMP requires GTP. An excess of ATP accelerates the pathway leading to guanylic acid, and an excess of GTP accelerates the synthesis of adenylic acids. A second type of coordination between AMP and GMP levels is provided by two feedback inhibitors, that of conversion of inosinic acid to adenylsuccinic acid by AMP and that of conversion of inosinic acid to xanthylic acid by GMP. The first of the processes regulating AMP VS. GMP levels is a rapid process in which GMP synthesis is accelerated when AMP levels are high, whereas the second is a slow process in which AMP and GMP retard their own synthesis (9).

In rapidly dividing cells, the CAMP concentration is relatively low whereas in cells undergoing cessation of proliferation, the cAMP concentration increased (10). Calcium, high doses of parathyroid hormone or alpha adrenergic agents administered to humans caused an increase in cGMP levels in human urine and plasma. Except for parathyroid hormone, these agents either did not elevate or lowered cAMP levels (11).

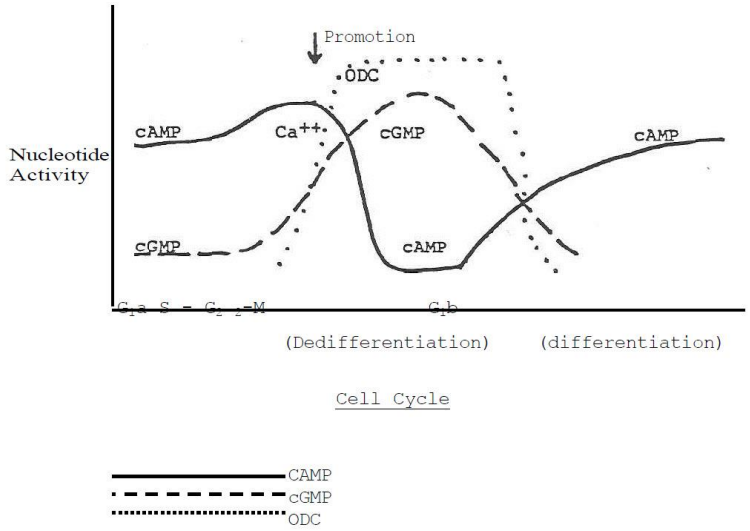
Mitotic cells have much lower cAMP levels than are present during the G1, S, and G2 phase or interphase. During progression through G1_, S, and G2 the cAMP levels stay approximately constant but as cells go into M it falls to about one half the concentration present in previous stages. In quiescent cells blocked in G1, the level of cAMP is much higher than that of the corresponding cGMp. The following is a hypothetical correlation of the cyclic nucleotide ratio during the cell cycle.

4.2 Tumor Promoters and Cyclic Nucleotides

Many tumor promoters cause rapid rise in cAMP, which could activate cGMP. These promoters also increase the uptake of hypoxanthine which in influenced by cGMP (12) (13). The affect of the phorbol ester tumor promoter TPA on cyclic nucleotides varied with different experiments, depending on tissues used and methods.

In mouse skin, TPA appeared to lower cAMP (14, 15) but this was attributed to skin preparation (16). Belman et. al., on the other hand, reported that a single treatment of mouse skin with the tumor promoter PMA does not alter the CAMP levels of the epidermis up to 72 hours (17).

The affects of phorbol diesters on cGMP is equally controversial. PMA and TPA increased cGMP in epidermis (18), and 3T3 cells respectively; however, Mufson in four separate experiments found no increase in cGMP in epidermis with TPA (19), He did find an increase in the activity of the low and high forms of cAMP phosphodiesterase activity following TPA. The magnitude of the enzyme induction correlated with the tumor promoting activity of several doses of TPA (20).



Animals fed asbestos showed a significantly lower CAMP level compared to controls (21). Skin carcinogenesis induced by 7, 12 dimethylbenzanthracene (DMBA) was actually enhanced by an intraperitoneal injection of cAMP (22).

Possibly the transient increase in CAMP with the Ca++ influx is essential for a catalyst type activation of cellular activity similar to a nerve induction with the Na+ influx reaching a peak and then dropping off once the activity is initiated. Another possibility is that the rapid influx of Ca++ acts on cAMP, which would explain the ODC activation, and then the elevated level of cAMP turns on cGMP activity directly or indirectly.

4.3 Transformed Cells and Cyclic Nucleotides

Many cancer cells have lost the control mechanism which sends nutritionally limited normal cells into G1_ quiescence, so when, for whatever reason, they do not divide, a spectrum of cancer cells blocked in all of the cell cycle is produced.

It seems that tumor cells cAMP is more likely concerned with the regulation of tumor cell function than of growth (23).

In many tumors, as well as in the plasma and urine of cancer patients there is often seen a reverse in the ratio of cyclic nucleotides. Although this usually effects cGMP through an elevation of its levels (24), lower levels of cAMP may also occur (25). This latter effect need not occur to reverse the ratio if the cGMP level exceeds the normal cAMP level. It is therefore the quotient of the cAMP to cGMP values in cell growth and transformation that is of importance rather than the individual values themselves (26, 27). In patients with disseminated cancer, cAMP values in plasma and urine did not differ significantly from those in normal subjects (28).

The following forms of cancer showed changes in their nucleotides;

	(56)	Tumor
(60)	(57)	Lung
(61, 62, 63)	(58)	(metastatic)
(64)	(59)	Colon (metastatic)
		Breast (metastatic)
		Acute Myelocytic
(65)		Leukemia
(66)		Hodgkins
		Gastric Cirrhosis
		Epithelial (general)
		Nevus-cell-nevus
		Basalioma Melanoma (malignant)
		Esophagus Brain (General)
		Medulloblastoma Glioma (brainstem)
		Cystic Astrocytoma

cGMP		**CAMP**
Lung	I:P&U	N/G
Colon	I:P&T	D:T
Breast	I:P	D:T
AML	I:P&U	D:T
Hodgkins	I:P&U	D:T
Gastric	I:P	D:T
Cirrhosis	I:P&J	D:T
Epithelial	N/G	D:U

Nevus Cell	I:T	D:T
Basioloma	I:T	D:T
Melanoma	I:T	D:T
Esophagus	I:U	N/G
Brain (General)	I:T	D:T
Medulloblastoma	I:CSF	D:CSF
Glioma	I:CSP	D:CSF
Astrocytoma	I:CSF	D:CSF

I= Increase
P= Plasma
CSF= Cerebral Spinal Fluid
D= Decrease
U= Urine
T = Tumor Tissue Fluid
N/G= None Given

The changes in extracellular fluid cyclic nucleotide levels in cirrhotic patients is very similar to those reported for primary hepatoma patients. Tumors induced by chemical carcinogens also show reversed nucleotide ratios. Colonic and small intestinal tumors induced in adult male rats by 1,2 dimethylhydrazine (DMH) (as well as x-irradiation) were found to have half the intracellular concentrations of cAMP (29, 30) found in normal large bowel tissue. The intracellular cGMP in the neoplastic tissue was twice that of normal colon (31).

4.4 Receptors and Cyclic Nucleotides

Stimulation of specific receptors in normal tissue effect the activity of the different cyclases responsible for elevations in their corresponding nucleotides. These cyclases can also be influenced directly in normal cells by certain hormones.

In transformed cells, there appear to be changes not only in receptors in some tumors, but also changes in the cyclases themselves. This latter effect is particularly noted in the response of cancer patients to glucagon, which acts directly on adenylcyclase rather than through a receptor. Normal patients showed an increase in plasma cAMP following an infusion of glucagon, whereas patients with malignancy failed to produce the same degree of elevation (32).

The shift in receptors, either alpha substituted for beta or vice versa, may partially depend on the tissue origin of the tumor. Usually the beta adrenergic system shows a decreased stimulatibility and the cholinergic system an increased one depending on the stage of the disease (33). This is especially noted when comparing adrenocortical carcinomas with C-6 glial tumors. The adrenal neoplastic cell possesses ectopic alpha adrenergic receptors. Epinephrine increased the cGMP in these cells which is blocked by the alpha-adrenergic antagonist phenlolamine. Neither acetyl choline, a cholinergic agonist, nor exogenous calcium caused an increase in cGMP (34).

The *C-6* glial tumors on the other hand appear to possess beta receptors that effect cGMP. When they were treated with Norepinephrine and sodium azide, there was a tremendous increase in cGMP that was mimicked by a beta agonist, isoproterenol, but

not by the alpha agonist phenylephrine. Propanol and sotalol, beta blockers, inhibited the increase of cGHP (35) . TPA prevented an elevation of cAMP following adrenalin in normal tissue (36). The presence of an endogenous activator of guanyl cyclase in neuroblastoma, NIE 115, cells was discovered by Amano et. al: it is inhibited by N-methylhydroxylamine and lysolecithin (37).

These factors may assist in the shift of the cells toward a dedifferentiation with an elevation of cGMP.

The other change affecting cyclic nucleotides in tumors is the activity of the phosphodiesterases which normally act to decrease their respective cyclic nucleotides.

4.5 Phosphodiesterase and Cyclic Nucleotides

In normal cells there appears to be a fine balance between specific nucleotides and the contrasting phophodiesterase. For example, the cAMP phosphodiesterase from normal lymphocytes is inhibited by the contrasting cyclic nucleotide, cGMP. cAMP phosphodiesterase from transformed lymphocytic cell lines or lymphocytic cells from leukemic patients, however, does not appear to be inhibited by the CGMP nucleotide as are normal cells (38).

In addition to the human lymphocytes inability to increase cAMP by inhibiting its cAMP phosphodiesterase, there is also a lack of cGMP phosphodiesterase in these cells. These abnormalities may be related to the defective lymphocytic growth control (39).

It is interesting that even the muscles of cancer patients showed an increased phosphodiesterase activity using cAMP and cGMP as substrate (40).

The increase in the cAMP phosphodiesterase of the epidermis by TPA may also assist in decreasing cAMP in this tissue, (41) and encouraging cellular division.

4.6 Inhibition of Nucleotides

It has been suggested that cAMP acts as an inhibitor of some phases of the cell cycle (42) thus decreasing the mitotic rate. Inhibition of mitosis is often accompanied by more extensive differentiation of cells.

Caffeine increases the cellular 3’ 5’ cAMP by inhibiting the cAMP phosphodiesterase. Theophylline and papaverine, cAMP phosphodiesterase inhibitors, not only increase cAMP levels but they depress the production of plasminogen activator (43). In mammalian cells, a variety of proteases are able to cause a drop in the cAMP level. Exogenous dibutyryl cAMP is able to block the growth stimulation by protease or by serum (44). cAMP has been shown to inhibit the growth of hormone dependent mammary tumors (45) when combined with theophylline, cAMP can inhibit mitosis (80%) in the mouse ear (46) and promotion by croton oil (47). Treatment with dibutyryl cAMP and theophylline inhibited both the tumor promoting and mitotic stimulating effects of TPA (48).

5’ AMP, cGMP and cAMP are all equally effective in inhibiting tumor development when injected at the same time as promotion with croton oil (49).

Spermine and other polyamines are capable of inhibiting cGMP phosphodiesterase activity in the bovine brain (50). This could explain a contribution of ODC in the

activation of cGMP in cells. The inhibitor can be overcome by Ca++ and calcium dependent regulatory protein in normal tissue (51). Calcium has little or no influence, however, on the proliferation of tumor cells indicating a loss of control.

An inhibitor of guanyl cyclase, GTP pyrophosphate lyase (GCI), was found in the balsam pear, momordica charantia. This extract blocked the growth of the rat prostatic adenocarcinoma and [3H] Thymidine incorporation into DNA at the G+2M phase of the cell cycle. This phase is the presumed locus of cGMP effects. The guanyl cyclase activity was higher in the tumor than in normal prostate tissue and was decreased in culture by the extract (52).

Another phosphodiesterase activity was demonstrated from disrupted leukemia L-1210 cells which was 3' 5' cytidine monophosphate (cCMP) specific (53).

4.7 Cyclic Nucleotides in Lymphocytes

Patients with malignant melanoma show an elevated B lymphocytes and a decrease of the T lymphocytes compared to controls. The cAMP and CGMP level in lymphocytes of patients suffering from melanoma shows a correlation to the degree of metastasizing changes (54). In the T lymphocytes of these patients there is a diminished activatibility of the CAMP level and a higher stimulatibility of the cGMP level (55).

5.0 Protein Kinases

Regulation of the biological activity of proteins through phosphorylation and dephosphorylation reactions has been established as one of the most significant mechanisms in-controlling a variety of cellular processes (1). One of these processes is the changes in susceptibility of proteins toward proteinases. This has been observed after covalent modification by phosphorylation and dephosphorylation (2). Phosphorylation is catalyzed by the protein kinases which are found in every mammalian tissue as well as bacteria (3). The activation of dependent protein kinases appears to be the way that cyclic nucleotides achieve their specificity in the regulation of the cell (4).

These kinases seem to be extremely specific, phosphorylating specific histone regions, or specific proteins (5). cAMP protein kinase catalyzes the phosphorylation of several proteins (6) including phosphorylase kinase, casein and protamine (7). The casein substrate seems to be preferred, however, by a cAMP independent kinase.

Yamamura et. al. found two protein kinases in the liver of rats preferentially phosphorylated histones. One was cAMP dependent, the other was cAMP independent referred to as Kinase B1_ and Kinase B2 respectively (8).

Another study also showed a cAMP independent rat liver cytosolic protein kinase, casein kinase TS, that displayed a remarkable specificity for the same sites of casein fractions as are phosphorylated endogenously by the mammary gland casein kinase (CK). There are also cAMP independent kinases found in the membranes structures such as mitochondria and microsomes (9, 10).

5.1 Kinase Specificity

Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) activate specific protein kinases (11, 12) that are not only select in their phophorylating activities (13, 14, 15, 16) but often mediate different and opposing biological actions (17). Their mechanisms of action and amino acid composition, however, are very similar (18). Kinase A, the cAMP dependent protein kinase, mediates most of the intracellular effects of cAMP (19, 20). In many tissues the concentration of Kinase A exceeds the cGMP dependent protein kinase, Kinase G, with the exception being the lung and cerebellum where the concentration of the two are more nearly equal (21). The cAMP dependent protein kinases activity is regulated by the disassociation of the haloenzyme into regulatory and active catalytic moieties in the presence of cAMP. It exists in mammalian tissue as two main isozyme forms termed protein kinase Type I and protein kinase Type II (22). The two subunits present in the Kinase A molecule, upon addition of CAMP, bind the regulatory subunit and allow the catalytic subunits to become active. The two isozymes described for Kinase A show a unique catalytic subunit, the regulatory subunit, however, is different (23, 24).

These kinases appear to be cell cycle dependent and show elevations dependent on the stage of the cycle.

In Chinese hamster ovary cells the Type I protein kinase was highest in mitosis and Type II was highest at the G1 boundary (25). The activation of the specific kinases

may be essential for the derepression and deregulation of DNA (26) depending on whether kinases are GMP dependent or AMP dependent.

The cGMP specific kinases may be the key enzymes that set cell division into motion (27) after the initial upsurge of Ca++ and CAMP. They may be essential for anabolism and mitosis leaving the other areas for catabolism and differentiation with the cAMP specific kinases.

5.2 Kinase Modification of Protein

The phosphorylation of seryl' and threonyl residues by specific protein kinases appears to be the most frequent device by which the biological activity of proteins can be modified (28). Both kinases phosphorylate acceptor proteins but vary in their kinetic and physical properties. There is a more general phosphorylation of histones by Kinase A, and a more specific histone phosphorylation by Kinase G (29).

Kinase G phosphorylates fewer substrates, mainly seryl and some threonyl residues of acceptor proteins in the arginine rich histones, but not the muscle glycogen phosphorylase kinase nor the glycogen synthetase (30).

Kinase A phosphorylates glycogen phosphorylase kinase, glycogen synthetase, ribosomal protein, membrane proteins and nuclear non histone proteins with no species specificity.

Histones may represent a unique substrate in that they induce dissociation of the subunits of cAMP dependent protein kinase and exhibits an interaction with a modulator which enhanced cGMP protein kinase activity (31). It has been suggested that protein kinase stimulatory modulator (KSM) is necessary for cGMP to stimulate phosphorylation by cGMP kinase.

Kinase A is much more effective in phosphorylating the arginine rich histones than the lysine rich histones. Kinase G, on the other hand, finds the arginine rich histones only about twice as effective as the lysine rich histones as a substrate (32).

The phosphorylation of histones by Kinase A affects their binding to DNA and may induce conformational changes. The phosphorylation of H1 histone increased the molecular mobility of H1 in H1-DNA complex, and loosened the binding of H1 and H5 to DNA (33).

Some kinase fractions phosphorylate non histone chromosomal proteins as well as histones in He La S3 cells. This phosphorylation was also cell cycle state dependent. In some fractions in the in vitro assay the histones influenced the phosphorylation of non histone proteins and vice versa (34).

5.3 Membrane Associated Kinase in Cancer Cells

A kinase located at the surface of cultured cells was found that was elevated in normal growing fibroblasts, but disappeared when they were contact inhibited. This kinase remained high, however, in transformed and malignant cells whatever their growth state (35).

Human epidema carcinoma cells show the activation of a membrane associated protein kinase by epidermal growth factor (EGF) that phosphorylated specific membrane proteins (36). One of the proteins phosphorylated is tyrosine (37). The

EGF receptors in epidermal cancer is increased whereas in bronchogenic cancer and rhabdomyosarcoma they are decreased (38).

Plasma membranes of transformed cells have a defect in an endogenous cAMP dependent phosphorylation system which can be corrected by addition of exogenous cAMP dependent protein kinase (39).

5.4 Prostaglandins and Kinases in Cancer

Prostaglandin E1 elevates cAMP in many types of cells and is important in thymus activity (40). Many tumor cells are unable to make PGE1 (41). PGE1 elevated cAMP accumulation in human gut adenocarcinoma (HT 29) cells, (42), neuroblastoma glioma hybrid cells (43), wild type Cloudman S91 melanoma cells (44), diploid and SV 40 transformed human fibroblasts (45).

In normal adrenal cells and adrenal tumor cells, PGEi stimulated the cAMP protein kinase activities. In one tumor in which the adenylcyclase activity was insensitive to ACTH, PGE1 stimulated cAMP protein kinase. Another tumor, that was insensitive to PGE1 showed an increase in protein kinase activity with ACTH. Dibutyryl cAMP, however, stimulated activity in both types of cells. A close link correlation was found between protein kinase activation and Cortisol production at the lowest concentration of ACTH (46).

5.5 Alterations of Kinases in Cancer

There are changes found in specific cyclic nucleotide protein kinases of leukemic cells as measured by the incorporation of p32 into histones. In both chronic (adults) and acute (children) leukemic cells. Kinase A activity was low and Kinase G activity was high in the absence of the respective cyclic nucleotides. Upon addition of cAMP and cGMP in vitro, value for Kinase A activity returned to normal whereas those for Kinase G increased further (47).

A significantly lower degree of cAMP dependent endogenous and exogenous protein (histone) phosphorylation was also observed in x-irradiation induced mucoid adenocarcinoma of Holtzman rat small bowel (48).

The ratio between protein kinase Type I and protein kinase Type II in renal carcinoma extracts was about twice that from extracts of normal renal cortex, although the total soluble cAMP dependent protein kinase activity was similar in extracts from normal and malignant tissue (49).

In response to estradiol and cardiac hypertrophy, altered distribution of the two main isozyme forms of cAMP dependent protein kinase have been noted during various phases of the cell cycle.

In one study, the increase in Type II protein kinase activity seemed to be related to the initiation of DNA synthesis is S phase (50) however, Defer et. al. suggested that the initiation of DNA synthesis occurs in the G3 phase involving chromatin phosphorylated proteins. In Reuber H-35 hepatoma cells (in vitro), the chromatin protein kinase activities increased in the late G1 phase and continued to increase in the S phase (51).

In hepatoma cell lines, a complete absence of protein kinase Type I and its associated high affinity binding protein has been noted (52, 53, 54).

The HT 29 cells contain one Type I and two Type II cAMP dependent protein kinases, plus one or two major and two minor cAKP independent protein kinases. The independent kinases do not disassociate upon maximal stimulation like the three haloenzymes (55).

Another species of protein kinase was found in Morris hepatomas that utilized endogenous proteins as phosphate acceptors that were tightly associated with the enzyme. The phosphorylation of these endogenous proteins was greatly stimulated by histone and protamine as well as by other polycations, polylysine, polyarginine and polyamines (56, 57).

5.6 Kinase Substrates and Cancer

Differences in phosphorylation of protein substrates are seen in various types of cancer including neoplastic hepatocytes, adrenocortical carcinoma (58, 59, 60, 61) and Ehrlich ascites tumor cells (62).

The hepatoma cells were found to phosphorylate arginine rich histones H3 and H4, and showed differences in the phosphorylation of histone H1. The c-terminal fragment of H1 was phosphorylated by hepatoma protein kinase, but not by normal liver protein kinase (63).

The cyclic nucleotides dependent protein kinases can be substrates for themselves. cGMP dependent protein kinase can be a substrate for its own phosphotransferase activity and other substrates such as histone and cyclic nucleotides modulate the process of self phosphorylation (64).

The adrenocortical carcinoma related protein kinase bound cAMP and autophosphorylated, but it did not use histones, casein nor polysomes as substrates in the presence of cAMP (65).

The time course of phosphorylation of eukaryotic ribosomal proteins from HeLa and Krebs II ascites tumor cells using cAMP dependent protein kinase and cGMP dependent protein kinase suggested four types of phosphorylation reactions: (66) protein (subunit) S 2, S 10 and L 5 were preferably phosphorylated by the cGMP dependent protein kinase; (67) proteins S 3 and L 6 were phosphorylated at very similar rates by either kinase; (68) proteins s 7 and L 29 were almost exclusively phosphorylated by the cAMP dependent protein kinase; (69) protein S 6 and most of the other proteins were phosphorylated about two to three times faster by the cAMP dependent than by the cGMP dependent enzyme. These results suggest that organization of several proteins in large particles may result in selective substrate specificity for cyclic nucleotide-dependent protein kinases in vivo.

Kinase specificity varies throughout the cell cycle. In the G1 phase of HeLa S3 cells, the kinase phosphorylated mainly high molecular weight polypeptides whereas lower molecular weight species are phosphorylated by kinases from the S, G2 and mitotic stages of the cell cycle. Inhibition of DNA synthesis by cytosine arabinoside did not affect the activity of substrate specificity of S-phase kinases.

5.7 Tumor Virus Associated Kinase

A specific protein kinase activity is associated with many virally transformed cells (70, 71, 72, 73, 74, 75). The avian sarcoma virus mediates the neoplastic transformation of cells by a single viral gene (SRC) which encodes a phosphoprotein (PP60 SRC) with the enzymatic activity of protein kinase (76). This kinase appears to be cAMP independent (77) and is similar to an endogenous normal phosphoprotein with possible protein kinase activity like the PP60 SRC. (78, 79, 80). It is postulated that this kinase is essential for transformation (81) and that phosphorylation may be involved in the transforming activity of viruses causing leukemia as well as sarcomas (82, 83).

An SRC virus antigen near the plasma membrane causes GTP antophosphorylation and threonine phosphorylation (84). This may be a kinase.

5 .8 Tumor Inhibition and Regression

Hormones can have stimulatory or inhibitory effects on tumors. This may depend on many factors including cell type, receptors and the stage of the disease. Insulin increases cGMP (85). A decrease in insulin is usually followed with an increase in glucagon which can stimulate cAMP (86). In DMBA induced mammary tumors, those responsive to the induction of diabetes had initially a fourfold lower level of cAMP than unresponsive tumors, but cAMP increased fourfold during regression (87). Unresponsive tumors did not show any statistically significant changes. The total histone kinase activity increased 127% in the cytosol and 153% in the nuclei within 36 hours after treatment. Mammary tumors regression due to diabetes seems to involve the cAMP system and occurs through a sequence of events similar to those observed during regression induced by either ovariectomy or dibutyryl cAMP treatment (88, 89).

An endogenous inhibitory factor exists that is specific for one of the soluble nucleotide independent casein kinases of the adrenal cortex. The kinase, CKG, uses GTP as well as ATP for a phosphoryl donor. Its inhibitor, CKGI, is quite specific.

Saturable binding by polyamines of CKGI is observed in addition to a direct stimulating effect on this enzyme. Spermine and other naturally occurring polyamine structures can reverse the inhibitory action of CKGI upon CKG activity (90).

In nuclei from regressing mammary tumors, a new non histone basic protein species of a higher molecular weight becomes the predominant substrate of the enodogenous kinase. Concomitant with the increase in phosphorylation of regression associated proteins is a decrease in the phosphorylation of growth associated proteins, an increase in cAMP binding activity and an increase in histone kinase activity in the regressing tumors (91, 92). The preferred exogenous substrates for protein kinase from growing and regressing tumor nuclei are casein and histone respectively, suggesting different kinases in growth such as casein kinase (cAMP independent kinase) and histone kinase (cAMP dependent kinase) in regression (93).

Morris hepatoma 3924A shows a decrease binding and responsiveness to glucagon, a decreased catalytic activity of adenylcyclase, altered responsiveness of adenylcyclase to prostaglandins, increased levels of membrane bound guanylate cyclase, increased cAMP phosphodiesterase, decreased cGMP phosphodiesterase and an increased phosphodiesterase modulator protein (94, 95).

In neuroblastoma cells treated with 8-bromo-adenosine 3'-5' cyclic monophosphate, the cAMP histone kinase is activated. A translocation of the kinase from cytosol to nuclei is seen which precedes the activation of tyrosine hydroxylase (96). Intact microtubules are required to transfer the catalytic subunits of activated protein kinase from cytosol to the nucleus (97, 98). After translocation, the protein kinase prefers acidic protein as a PO 43 acceptor rather than histone (99). In rat pheochromocytoma, tyrosine hydroxylase is a substrate for cAMP protein kinase (100).

6.0 Protease

Normal cells generally secrete some granule bound proteases. A number of proteases with extremely high substrate specificity are known that can select substrates for degradation without any additional control mechanism. Some examples of highly specific proteases are provided by: the group specific" proteases from mast cells that inactivate apoenzymes derived from pyridoxal phosphate dependent enzymes; and "rec A gene products from E Coli which promotes proteolytic cleavange of bacteriophage (lambda) repressor. Calcium binding proteins control the calcium activation of protease (1, 2).

Activation of plasminogen by limited proteolysis produces extracellular proteolytic actions including fibrinolysis, virus and carcinogen induced oncogenic cellular transformation and inflammatory responses. Urokinase, tissue activators, streptokinase, nerve growth factor and other proteinases all activate plasminogen (3, 4). A protease purified from human skin induced leukocytic infiliation upon injection into skin. The enzyme was a serine protease, but not plasminogen activator (5).

Every form of cancer except that of the blood forming tissues is characterized by the extracellular secretion of abnormally large amounts of proteolytic enzymes. Within growing tumors as well as in cell cultures, its major action appears to be proteolytic activation of the inert serum protein "plasminogen" to form plasmin, a proteolytic enzyme (6). It is called plasminogen activator, and appears to be a serine esterase. plasminogen activator increases sugar transport and saturation density in the cell (7).

6.1 Plasmin

Plasmin, trypsin and kallikrein are serine esterases (8). Plasmin resembles trypsin (9, 10), the most important digestive enzyme of pancreatic secretion (11). Trypsin catalyses the hydrolysis of only those peptide bonds in a polypeptide chain whose carbonyl function is donated by either a lysine or an arginine residue, regardless of the length or amino acid residue of the chain (12). In vitro, trypsin removes more than half of the protein groups that protrude out of the lipid bilayers (13). Glycoproteins from the cytosol of a variety of mammalian cells are more rapidly degraded than non glycoproteins in every molecular weight and isoelectric point class (14).

In non malignant mouse fibroblasts, trypsin, ficin or pronase added to the confluent cultures caused cell division and escape from contact inhibition. Plasmin caused 3T3 cells to express transformation like features even though they did not become transformed (15).

In E. coli an inhibitor to ornithine decarboxylase has been found that is labile to trypsin (16). A masked protease localized in the cell wall of E. coli is activated by solubilization with deoxycholate (17). In bacteria there appears to be degradation of an endogenous ornithine decarboxylase inhibitor by a protease which may be activated by a bile salt. A trypsin like protease has been described associated with ribosomes and polysomes of HeLa Cells and the ribosoraes from rat liver (18).

When microsomes are exposed to deoxycholate, the endoplasmic reticulum membranes are solubilized and the free polysomes of normal liver are then found to possess activity equal to those of regenerating liver. Pretreatment of normal

microsomes with trypsin also tends to eliminate the difference shown to derive from inhibitor(s) present in the microsomal membrane fraction of normal liver (19, 20).

6.2 Protease Activity in the Cell

Protease is capable of degrading histones, one of several nucleoproteins inhibiting transcription (21). The removal of histones by proteolysis is important during the transformation process (22).

An enzyme found in the microsomal fraction of rat liver hydrolyzed basic proteins such as histones, protamine and slightly ribonuclease (23).

The separation of histones from DNA in vitro depends on both the cations and anions constituting the salt solution. The efficiency decreases in the order of: deoxycholate CIO4 Cl- SCN F-

Trypsinized nucleoli biosynthesize an RNA which resembles DNA in its base composition. The removal of histones by trypsin, however, does not activate the polymerase enzyme (24).

The appearance of proteases in hormone stimulated cells is initially seen in the cytoplasm and later in the nucleus (25). Either the protease is travelling into the nucleus where it appears to be activated, or else there are several proteases involved with their activation dependant on the cytoplasmic protease. Data from ratuteri seems to support the former theory.

In bacteria, damage to DNA results in the simultaneous induction of a number of functions called “SOS functions” after the international distress signal. TWO of these SOS functions are of primary importance for carcinogen testing: (a) the induction of error prone DNA repair, and (b) the induction of the lambda prophage (26, 27). Demonstration of a proteolytic derepression of the genome is seen in the requirement of protease for error prone DNA repair (28). Further studies with protease inhibitors substantiates the participation of the proteolytic processes in the induction of an SOS system of DNA repair (29).

There are several genetically determined diseases associated with DNA repair defects, thought to be the cause of cancer sensitivity. The following examples: Fanconi’s Anemia, Bloom’s Syndrome, Ataxia Telangiectasia and parakeratosis Mibelli are relatively well known. In XP, the oversensitivity of the skin to U-V light is inherited and the condition often leads to skin cancer (30).

In bacteria, caffeine inhibits excision type DNA repair, and shows a synergistic killing effect with U-V irradiation. A similar effect on survival is seen in rodent cells in culture, but excision repair is not affected.

Normal human cells in culture, however, show little effect of caffeine either with or without UV-irradiation; whereas a strain of fibroblasts from a xeroderma pigmentosum patient (XP-variant) which shows normal excision repair is susceptible to the synergistic killing effect of caffeine after UV-irradiation. Caffeine also potentiates uv-induced cell mutagenesis in the latter strain (31).

Unfortunately, the species specificity of exogenous influences on DNA repair makes the extrapolation of test data for carcinogens and mutagens from animals and bacteria to man somewhat ambiguous.

6.3 Hormonal Influence on Protease

Protease activity is influenced by hormones, such as testosterone and estradiol. Testosterone increased the activity of protease in the submaxillary gland of castrated rats. Spermidine synthesis may be necessary for the stimulation of protease synthesis by testosterone since the polyamine spermidine also increased protease activity, although to a lesser degree (32).

Estradiol given to ovariectomized mice resulted in the appearance of plasminogen activator in the uterus, (33) which first appeared to be associated with the plasma membrane and later appeared in the nucleus (34). This protease hydrolyzed specific protamines (arginine rich proteins) as well as specific histones in the nuclei (35). Antipain and leupeptin, protease inhibitors, inhibited the estradiol induced protease and inhibited DNA synthesis and function (36).

Immunofluorescence studies provide evidence for a structural labilization of lysosomal constituents and their translocation to the nuclear compartment after the administration of estrogen to ovariectomized rats (37). Target tissues respond to hormonal stimulation, in part, by the biosynthesis of new species of RNA which in turn code for new protein species. In rat uteri, estrogen treatment of the animals causes an increase in the labelling of non-histone proteins which partly occurs within thirty minutes after the administration of the hormone, secondary to the RNA synthesis. The RNA appears to accumulate as soon as the hormone enters the nucleus. The chromatin DNA sites stimulatated by the hormone treatment were those which were also sensitive to trypsin indicating an apparent competition of estrogens, or their associated protease(s), for similar sites in the uterine chromatin (38).

6.4 Protease in Tumor Promotion and Induction

Mediated by hormones and other effectors (39), different proteases may be acting in the initiation and promotion process (40). Studies with both phorbol esters and ingenol tumor promoters have demonstrated a consistent correlation between tumor promoting activity and induction of plasminogen activator (PA) in cell cultures (41, 42, 43, 44). The earliest changes by phorbol esters are increases in protease activity and ornithine decarboxylase (45, 46). TPA has been shown to alter the promotion composition of the outerside of the membrane and induce surface structural changes in the surface of lymphoblastoma cells (47). TPA also enhances error prone DNA repair at the endonuclease step (48).

Janoff et. al. found that small amounts of the phorbol ester PKA, produced a severe local vascular reaction in the Ekin (ear) of CF and STS mice, followed within 24 hours by an intense leukocytic infiltration and marked increase in tosyl arginine methylesterase (neutral protease activity) (49, 50). The source of the increase protease activity to an influx of hydrolase rich phogocytic cells into the target tissue (51).

TPA enhanced PA production in hamster cell lines, exhibiting a high degree of specificity in the type of PA evoked, which is always of the lung form. No enhancement of PA production was detected in hamster cell lines synthesizing PA of different antigenic forms (52).

Induction of PA by TPA and PMA appears to require transcription (53, 54), since the increase is after a time lag indicating a m-RNA intermediate for its induction (55).

Increased levels of PA frequently accompany oncogenic transformation (56, 57, 58, 59, 60, 61). Transient PA induction has been shown to occur during transformation of hamster embryo cells by benzo(a)pyrene (62).

The expression of PA in RSV transformed cells is controlled by both the virus and the cell (63). Embryonal mouse fibroblasts transformed by RSV contained five serine enzymes not found in normal cells medium. TWO of these enzymes were PA. (64).

Wilson et. al, found that sarcoma virus transformation sensitizes chick embryo fibroblasts by lowering the threshold concentration for response to the action of PMA. The effect of transformation and PMA on PA synthesis are synergistic rather than additive (65).

Phorbol esters also produced synergy in induction of fibrolytic activity and membrane glycoprotein shifts which occured in chick embryo fibroblasts after transformation by Rous Sarcoma Virus (RSV) (66, 67).

RSV transformation, PMA, and retinoic acid all induce high levels of PA production by differentiating myotubes in the absence of DNA synthesis (68).

Under certain conditions B-all-trans-retinoic acid (RA) which inhibits ornithine decarboxylase (69) may have cell activating properties. RA treatment of Simian virus 40 transformed 3T3 cells resulted in a 5 to 6 fold enhancement of PA. Extracellular PA levels of Ra treated cells incresed by 4 to 10 times the amount of increase observed for Ca++, PMA or fraction 1 from lactalbumin hydrolysate (Fl) alone (70).

RA at low concentration induced multiple phenotypic changes in embryonal carcinoma cells lines including elevated levels of PA, production sensitivity to cAMP compounds and an increase synthesis of collagen-like proteins. RA induces differentiation of embryonal carcinoma cell into endoderm (71).

There appear to be different PA esterases, or activations of these esterase, in relation to specific tumors or tissues involved which may correlate with embryological origin of the tissue or the differences in m-RNA transcribed. For this reason Retinoic acid may enhance tumors in one type of tissue — e.g. mesenchymol — and inhibit in others -- e.g. skin.

6.5 Proteases and Cancer

A variety of proteases are associated with different forms of cancer. The protease plasminogen appears to alter the cell morphology. When cancerous mouse cells were grown in serum containing medium, from which plasminogen had been removed, they were still able to grow normally but they no longer had the distinct cancer cell morphology and looked as if they were almost normal (72).

Mammary tumors produce much more plasminogen activator than normal tissue in vivo and in vitro. Distinct differences were found in the response of enzyme synthesis to hormones by mammary tumors (73). In adenocarcinoma of the breast, a cathepsin-B like thiol proteinase dependent on de novo synthesis was found to be secreted in extremely high levels (74).

In a study by Jensen et. al. of human cervical epithelial, 50% of the dysplastic and carcinoma in situ cells contain high levels of cell associated serine protease. 40% of the morphologically normal metaplastic cells also exhibited high levels of plasminogen (75).

Human colon cancer exhibits significantly higher levels of plasminogen activator content than surrounding normal tissue, with the highest levels in tumors that showed

invasive propagation on metastic spread. Activator activity of the tumor extracts was completely inhibited by rabbit antibody to against human urokinase. The activity of normal mucosae was variably inhibited, suggesting the presence of several kinds of activator in normal tissue (76).

Plasminogen activator from lung cancer also showed an elevated urokinase like enzyme, however, unlike other organ cancer it did not seem to show any significant correlation between the histopathological character of the tumor and the activator content, or between invasiveness and activator content (77).

A rhabdomyosarcoma strain culture fluid from human neoplastic origin contained eight serine enzymes including four plasminogen activators. Human melanoma cultures contained two plasminogen activators out of seven serine enzymes that were the same as two of the rhabbo plasminogen activators (78). In metastic cells from C 57 BL/6J mice with melanoma, the production of plasminogen activator was higher than in cells that were not as metastic (79).

Melanoma secreted plasminogen activators immuno-chemically distinct from urokinase. Enzymes with similar characteristics have been observed in serum free harvest fluids collected from other neoplastic tissue (a breast carcinoma, a glioblastoma, a malignant teratoma, a uterine sarcoma and a carcinoma of the renal pelvis) and from normal tissue (8-week old embryo fibroblasts, normal esophageal fibroblasts, and one culture of normal adult bladder epithelium) (80).

Patients with bladder cancer who did not respond to neocarzinostatin (NCS) therapy showed proteolytic activity in their serum which degraded NCS (81) Spitsyn et. al. also found an increase in protease activity of blood serum from patients with gastric cancer (82).

The PA produced by human brain tumor cells is antigenically different from the plasminogen activator of other human normal and neoplastic cells (83). In glioma cells, the most intense fibrinolytic activity coincides with mitotic waves (84).

An increase in serine esterase enzymes is seen in other pathological conditions aside from cancer, such as pancreatitis (85).

Since proteases can be quite specific, the differences seen in various cancers is not all that surprising as they are most likely involved in many normal endogenous cellular activities on a regular basis as well as in inflammatory activity.

6.6 Inhibition of Protease

Proteases can be inhibited by various mechanisms, either direct as in the action of leupeptin, or indirect as with the steroids which elaborate an inhibitor or the phosphodiesterase inhibiters which elevate cAMP.

Tumor promotion by phorbol esters and the inflammatory response were inhibited by the synthetic protease inhibitors tosyl lysine chlormethyl ketone (TLCK) and tosyl phenylalanine chlormethyl ketone (TPCK) (86, 87).

The microbial peptide leupeptin inhibits the increased activity of p-toluene sulfonyl-l-arginine methyl esterase seen after the application of the phorbol crotin oil (88). Leupeptin inhibited tumor promotion (89, 90, 91) in the colon, esophagus and mammary glands of rats and in the skin of mice. It did not, however, inhibit tumor formaton in the liver or forestomach of rats or the lung of mice and it enhanced tumor growth in the glandular stomach and bladder of rats. Enhancement of liver tumorigenesis by leupeptin occurred when it was applied during the promotion stage.

The effects of protease inhibitors on carcinogenesis varies with the schedule of administration (92). If early application of leupeptin inhibited DNA repair in general and late application inhibited abnormal or error prone DNA repair this might explain the differences dependent on various schedules.

Whether tumor promotion inhibitory doses of leupeptin also inhibit plasminogen activator in vivo is not known. After TPA treatment in vivo, only induction of partially characterized proteinases has been shown to occur (93). In chick embryo cells, however, TPA induced plasminogen activator (94, 95) but this may be only because it is embryo tissue.

Plasminogen activator was inhibited by dexamethasone in hepatoma cells (96, 97, 98) and by glucorticords in mouse peritoneal macrophages, rat tongue organ culture, rheumatoid synovial cells, human polymorphonuclear leukocytes and primary cultures of adult rat liver cells (99). Glucocorticoids inhibit promotion (100, 101, 102). Dexamethasone suppresses protease activity by elaborating a protease inhibitor (103, 104) via de novo RNA synthesis (105).

Variant cells have been isolated previously that are fully resistant to the dexamethasone inhibition of plasminogen activator activity. The basis for this resistance appears to be the failure of dexamethasone to induce an inhibitor (106, 107).

Dexamethasone and the protease inhibitor TPCK inhibit intiation by dimethylbenzanthracene (DMBA) during the early period of initiation and enhance initiation during the late period. Dexamethasone also inhibits prostaglandin (108).

Inhibition of error prone DNA repair is seen with elevation of cAMP and with protease inhibitors. An increase in cAMP is followed by a decrease in protease (109). Caffeine and theophylline were shown to inhibit ultraviolet mouse skin tumorigenesis (110). An increase in cAMP by theophylline, the phosphodiesterase inhibitor (111) or choleratoxin (112) will depress the production of plasminogen activator from Rous Sarcoma Virus and PMA (113). cAMP inhibited the growth of two hormone dependent mammary tumors (114).

Slaga et. al. found a combination of steroid and Vitamin A at non toxic levels produced greater inhibition (115). It is interesting that this combination will inhibit ornithine decarboxylase (116), and protease (117, 118) as well as prostaglandins (119). Inhibition of each area alone has been related to an inhibitory effect on tumor promotion suggesting further research utilizing a multi-factoral approach to cancer Inhibition.

7.0 Histones

Histones seem to affect the DNA activity in such a way that they inhibit or help regulate transcription. When histones are bound in vitro to DNA on a 1:1 ratio there is total inhibition of transcription (1). In cellular activity they are altered by protease and various other enzymes that phosphorylate, acetylate or methylate. Even the non histone proteins are affected by these activities. The alterations of histones induced by some carcinogens or in cell division are most likely affecting the susceptibility of DNA to mutation, or damage.

7.1 Histone Nomenclature

Histone nomenclature has many variations according to the individual investigators. For the sake of clarity, H1, H2A, H2B, H3 and H4 will be used to describe the individual histone fractions. The following chart describes the other nomenclatures.

Fraction

Lysine-rich					Arginine-rich
H1	H2B	A	B	VLR	SLR
F1	F2B				
La, lb	Iia, I	E2	E1		
I	Iib2				
Alfa2, a3	0.8s	1,1',1"	3		
H2A		H4	H3		
		F2a1	F3		
F2a2		IV	III		
		IV	III		
IIb		Beta	Beta		
		B	C		
		9	5		
IIb1		Beta 7	Beta 5		
		E1	E3		
1.6 s		5, 5'	2, 2', 2"		
		GAR	AR		
B		(127 p. 10)			
6, 7, 8					
Beta 6					
E1					
4					
AL					

7.2 Structure and Location

In cells of higher organisms the genes ore arrayed on chromosomes. Chromosomes are nucleoproteins: complexes of nucleic acids and proteins (2). Chromosomal material, chromatin, contains five complex proteins called histones that are positively charged and enriched in arginine and lysine, but completely lacking tryptophan (3) . They complex with DNA in a repeating pattern termed the nucleosome (4). The large number of lysine and arginine residues in the NH2 termini, as well as other regions of histones, together with evidence that all the lysine residues of histones are associated with DNA phosphates indicates that the NH2 termini is one of the histone regions that binds to DNA (5). DNA is on the outside of the nucleosome wrapped around the histones, which are located in the large groove of DNA (6, 7, 8).

A nucleosome contains a total of eight histone molecules (B2A, H2B, H3, H4)2 made up of equimolar amounts of the four types. The eight histone molecules are in association with approximately 140 base pairs of DNA and make up what is called the "core particle". H3 and E4 are associated with the ends of the core particle. The remaining DNA is variable in length among cell types and is termed the linker region which appears to be associated with Hl histone (9, 10, 11).

Histones are responsible for the compaction of DNA within the nucleus (12) and are involved in the maintenance of chromatin structure as well as in the repression of DNA-dependent RNA synthesis (13).

Although of the same class, the histones differ in primary structure that are referred to as subtypes or variants. Sequence differences between subtypes may range from a change of a few residues to massive substitution within inner histones. There are significant evolutionary divergences, and within a particular class of an inner histone from a single species, there are subtypes that have different primary structure. The most variable histone in H1; its weight as well as sequence are variable even within the same organism (14).

7.3 Histone Modifications and Interactions

The four core histones undergo five major types of covalent modification: acetylation, phosphorylation, methylation, ADP-ribosylation, and in the case of histone H2A, covalent linkage to the protein ubiquitin (15).

Ubiquitin is a very highly conserved protein that has been found in a variety of animal cells, bacteria, yeast and higher plants. Protein A 24 is a modification of the histone H2A. The epsilon amino of lysine 119 of the H2A is conjugated by an isopeptide linkage to the carboxy terminus of another peptide chain. The amino portion of the chain is the protein ubiquitin (16).

A24 is found in nucleosomes and inhibits transcription of DNA as does H2A alone (17). The amount of A24 has an inverse correlation with activity; it is decreased in regenerating liver and in acute lymphocytic leukemic cells (18, 19).

The formation of high molecular weight aggregates of histone is referred to simply as an aggregation and the formation of oligomers are called complexing. Histone-histone coroplexing occurs between the globular carboxyl regions of the inner histones. When histones aggregate, their secondary structure changes, and such a change implies tertiary structure changes as well (20).

Strong interactions occur between H2A and H2B and between H2B and H4 to form diraers; H3 and S4 interact to form a tetraroer, enzymatic reactions modify histone structure and can alter the interactions of histones with DNA (21).

Highly defined cross-link of histones occur (22) and can be induced by U-V light at position 26 of histone H2A and a tyrosine of H2B. (23, 24). H2A and H4 can bind to H2B simultaneously because of different binding sites (25). H3, H2B, and H2A can all form simple complexes with Hi (26). It is thought that a particular histone binding region may oscillate between the two DNA strands and that every core histone can be cross linked to the core DNA, (27) DNA unwinding requires that histones be transiently associated with single-stranded DNA (28, 29).

The histone content of normal cells that are active or inactive is the same. They block transcription by binding on to DNA rather than inhibiting the RNA polymerase. The selective removal of histones from isolated nuclei increases the rate of RNA synthesis indicating histones normal function is to inhibit the capacity of genes to be transcribed into

RNA. The messenger RNAs that code for histones are only associated with the cell's protein synthesizing organelles and translated into proteins at the time of DNA synthesis (30).

Histones and DNA are synthesized simultaneously.

Inhibition of DNA replication is accompanied by an immediate shutdown of histone synthesis and inhibition of histone synthesis results in a decreased rate of DNA synthesis (31, 32).

7.4 H1 Histone

Histone H1 is localized at the point where DNA enters and exits from the core particle. It seems as though Hl binds to one side of the core particle and both protects and locks in position entering and exiting DNA strands (33). The H1 subtractions can bind differently to DNA (34).

The carboxy terminal for H1 is highly basic and lysine rich (35, 36). It is the histone most susceptible to proteolysis. Of the core histones H3 is the most susceptible to proteolysis second to H1 (37).

Different Hl subtypes can interact both strongly and selectively with the non histone chromosomal proteins HMGl and HMG2 (High Mobility Group).

Studies of H1 depleted chromatin have demonstrated that readdition of histone Hl leads to chromatin contraction (38, 39).

A new minor H1 histone called Hlh was found in human tissue (40), which showed altered levels in some tumors.

7.5 Histone Phosphorylation

A mechanism that functions in the modification of chromatin trancriptional activity is the enzymatic phosphorylation of histones. The proteins of ribsomes; and RNA polymerase can also be phosphorylated in addition to phosphoproteins and histones with increases in their activity (41, P. 84-88).

Histone phosphorylation decreases the degree of template inhibition in the in vitro DNA polymerase reaction (42).

The phosphorylation of histones varies during the cell cycle with maximum levels in mid S-phase (43, 44, 45), the phosphates are attached through an ester bond to the hydroxyl residue of serine and to a lesser extent of threonine (46).

In mammalian cells H1, H3 and H2a are all phosphorylated; the phosphorylation of H2a is constant throughout the cell cycle, but that of H3 and H1 varies with the stage. Phosphorylation of H1 occurs just before mitosis, on different specific sites dependent on the kinase. Addition of phosphokinase from Ehrlich ascites tumor cells shifted mitosis to earlier times, indicating phosphorylation as an important step in mitosis (47).

Ord, Stocken and Kleinsmith have shown the p32 phosphate incorporation into rat histones occurs in vitro and in vivo with the main acceptor of the phosphate being the lysine rich H1 histone (48, 49). Inhibition of DNA synthesis has little effect on the phosphorylation of the lysine rich histone (50).

The intercation of DNA with histones changes the DNA conformation, and specific histone kinases change the physio-chemical properties of chromatin in non-dividing cells (51).

Langan established that the phosphorylation of histones was enzymatically performed by a histone kinase and a less specific phosphoprotein kinase both present in the cytoplasm (52). At least two kinase enzymes exist which are specific for different sites on the H1 histone (53, p. 84-88). The phosphoprotein kinase associated with chromatin are heterogenous.

Glucagon and insulin affect the rates of histone phosphorylation. cAMP dependent kinase and cGMP dependent kinase phosphorylate H2B with different primary sequences around the two phosphorylation sites indicating high specificity (54, 55). CAMP also stimulates the phosphorylation of H1 histone in vivo, in the presence of low concentrations of histones, CAMP dependent enzyme disassociates into a converted enzyme that is cAMP independent (56).

Phosphorylated H1 histones affect the DNA conformation considerably less than dephosphorylated hiiliones. X-irradiation decreases the phosphorylation of H1 histone and the amount of H1 histone kinase (57 f p. 85).

The extent of histone phosphorylation correlates with the promoting activity of phorbol esters and dose dependence paralleled promotion ability. Rainer et. al. reported a peak of histone phosphorylation two hours after treatment with TPA (58, 59). The phosphorylation of histone H1 by TPA was initiated at about the same time as the synthesis of DNA but past the S-phase (60).

The effect of mengovirus infection of Ehrlich ascites tumor cells was a dephosphorylation of H1 histone indicating an inhibition of the cells DNA synthesis (61).

Inhibition of histone H1 phosphate phosphatase activity by (lOmM) ZnCl2 lead to hyperphysiological levels of H1 phosphohistone in metaphase (62, 63).

In DMBA induced mammary carcinomas, dibutyryl cAMP treatment or ovariectomy produce a change in protein substrate specificity of nuclear protein kinase (64) and an increase in its activity in the regressing tumor (65).

Dexamethasone which elaborates a protease inhibitor, caused phosphorylation of H2A histone in both G1 and S-phase, however, this modification is thought to be a result of non-histone proteins (66). Perhaps the protease inhibitor is coded in the H2A associated DNA.

7.6 Histone Methylation

Histones as well as DNA can be methylated. The histones appear to shield DNA from methylation with the exception of H1 (67, 68). The two sites of H3 methylation are adjacent to the sites of H3 phosphorylation (69) . Histone methylation occurs after their biosynthesis and methionine acts as the donor of the methyl groups to lysine through an S-adenosyl-methionine intermediate. Once a histone is methylated it remains methylated (70).

In Chinese hamster ovary cells the H1 histone was not methylated, however, H1 histone is the major acceptor for ADP-ribose groups (71). The methyl content of H2B histone was minimal during Gl, rose rapidly during the early S-period, and then declined during mitosis. The main transfer of methyl residues to chromatin histones occurs during the profound, morphological changes of chromatin condensation. The methylated basic amino acids seem to promote structural changes in chromatin architecture (72, p. 90).

Arginine, histidine and lysine are the main amino acids methylated. They are methylated at the highest level in embryonic tissue and in older animals as well as in neoplastic livers (73, 74, 75).

Hypermethylation may be a consequence of increased enzymatic transmethylation or spontaneous chemical methylation. In the latter reaction, formaldehyde is the foremost agent suspected of being formed either from nitrosamines or as a result of demethylase activity in the presence of aromatic hydrocarbons or viruses in different tissues. Increased levels of methylated nucleic bases have been found in the urine of cancer patients compared with healthy controls (76).

An increase in methylation of histones is seen following the administration of different chemicals and carcinogens, such as nitrosomine and even methionine in some cases, and a decrease in methylation was seen with other “carcinogens”.

Modification of chromosomal proteins to produce methylated derivatives of lysine and arginine have been observed after the administration of 1,2 dimethyl hydrazine (77). Some of the intercalating anti tumor drugs (e.g. pyrido 4-3b carbazole congeners) also stimulate in vitro methylation (78).

Histone methylation was inhibited by the aminofluorenes with the degree of inhibition correlating with their carcinogenicity. The histone arginine methyl transferase also showed susceptibility to inhibition by adenine and the adenosine analog tubercidin as well as the DNA binding dyes ethidium bromide and acridine orange (79).

All carcinogens do not show the same mechanism of action and perhaps those carcinogens that increase methylation may activate endogenous substances which in themselves can be carcinogenic.

All three methylated basic amino acids were found in the Novikoff hepatoma arginine rich histones (80, p. 89). Purified enzymes from Krebs 2 ascites tumors cell chromatin catalyzed the transfer of methyl residues to protein (81, 82) in the presence of added histones. Both the histones and the non-histone proteins became methylated, epsilon N-trimethyllysine being the chief product of methylation which appeared to be 2/3 of the labelled protein not associated with histones (83). The presence of E-N trimethyllysine has been found in histones from calf thymus and chicken erythocytes (84, p. 89-90).

It was reported to have tumor growth enhancing effects in mice with NK/Ly ascitic tumors (85). Either this is one of the endogenous growth factors in some tumors or the experiment was not valid, since histones themselves can increase growth in some situations.

In the carcinoid syndrome, increased plasma concentration of the methyl donor methionine were reported. The plasma methionine levels returned to normal when serotonin production by the tumor was reduced 60% by parachlorophenylalanine (86).

Human acute lymphoblastic leukemia cells and other forms of tumor cells show a definite need for methionine, however, neither the elimination of methionine nor methionase were sufficient in all cases to destroy the tumor (87) since other pathways were then used.

7.7 Histones in Cancer

Histone abnormalities are noted in many forms of cancer. They are often associated with proteases that will degrade or alter specific histones. The altered histones and histone like proteins are found not only in the tumors themselves but also in the blood plasma. It seems as if the histones are synthesized rapidly but degraded even faster.

The major changes appear to be with H1 and H3 respectively. These are the histones most susceptible to proteases. H1 histone is also phosphorylated following tumor promoter TPA. The tumor promoting phorbol esters also stimulate histone synthesis (88). Baird et. al. showed that the ability of phorbol esters to stimulate RNA synthesis correlates with their tumor promoting ability (89). preparation of RNA and histones depress the electrophoretic mobility of lymphoid cells and oxygen consumption (90, 91).

In Morris hepatomas, as well as nitrosomorpholine induced hepatocarcinogenesis there is a decrease in the H1 lysine rich histone, suggesting an alteration in the binding of histones to chromatin (92, 93). In the metastases of the nitrosomorpholine induced hepatomas, there is a complete disappearance of histone H1 (94).

The H1 histones in BHK cells are synthesized in molor excess relative to the other histones during the Gl phase of the cell cycle, indicating higher concentrations of RNA for H1 in Gl (95, 96).

In mice bearing subcutaneous tumors, H1 histones are synthesized rapidly in the spleen. This stimulation is different from a general immune response (97).

In cancers, the amount of H1 was increased and the H1a, a minor H1 histone, decreased with one exception in hepatocellular carcinoma where the Hla was increased. In two cases of breast cancer, the Hla was decreased to an almost undetectable level (98).

In human chronic lymphocytic leukemia (CCL) there exists a histone fraction between H1 and H3 similar to Hla found in different human normal tissue and in the course of different diseases. The CCL cells also have higher levels of H3 and lower levels of H2B (99). Histone like proteins were isolated from both normal and tumor rat blood plasma: Hi, H2B and H2A, for normal plasma and H1, H3, H2B and H2A and H4 for tumor rat blood plasma (100, 101).

Virus induced cancers also show an abnormality in histones. A distinct change in microheterogeneity of histone H1 was detected after megovirus infection of Ehrlich ascites tumor cells (102).

Viruses themselves contain histones. H1 histones of animals exhibit a preferential interaction with circular superhelical virus SV 40 DNA as compared with open relaxed circular DNA (103, 104). The Simian virus, (SV40) DNA-histone complex lacks histone Hi (105). SV40 DNA is superhelical and H1 will interact with the viral DNA in a reversible manner (106). Changes in DNAase and RNAase are seen in viral leukemias. Liver and spleen cells from Rauscher's viral leukemic mice exhibited decreased nuclear acid DNAase and increased cytoplasm's acid and alkaline RNAase activities as compared to normal cells (107).

Hamster kidney cells (HK 21/C13) cultured with histones from rat liver or calf thymus, showed behavior similar to premalignant cells (108, 109). They showed invasive qualities in vitro, (110) and when injected into hamsters caused tumors that were also invasive (111). These activities from histones were not a result of the cationic nature of histones since polylysine and polyarginine did not cause the same effects (112). Injection of histone before the introduction of tumor cells enhanced their action, whereas if introduced after the tumor they showed inhibitory properties (113, 114, 115). This was not seen with all oncogenic viruses. Histones in vitro with tumor cells were cytolytic, apparently binding to the membrane (116). Perhaps in vivo their inhibitory action was partly immunological.

7.8 Protease and Histones in Cancer

Different proteases have been found in cancer and normal cells that act on histones and non-histone proteins. Some of the proteases found mimicked trypsin in their actions and showed specificity to certain areas on chromosomes. Endogenous proteases have been found to cleave at least H2A and H3 in the same region as trypsin (117). The histones in the puffs of polytene chromosomes were more sensitive to tryptic proteolysis than the histones in the condensed, non-puffing chromosomal regions (118, p. 141). Some of the proteases are actually non-histone proteins. Among the chromosomal non-histone proteins is a neutral protease which attacks histones in normal stable cells, but not the histones from the chromatin of which it is a component in regenerating liver (119).

Weintraub et. al. demonstrated that quite a stable digestion product, following proteolysis, exists in which each of the inner histones loses 20-30 amino acids from its N-terminal region, the putative DNA binding sites which contain about one third of the histone positive charge (120). One protease has been found that cleaves histone H2A near the sites of its potential joining to ubiquitin (121, 122).

Central prostate nuclei show differences in partially degraded histones from growing or dividing cells and from their castrated controls suggesting a differing distribution of histone proteases and/or modification of H2A. Limited selective proteolysis of histones in nuclei from regressing, hypertrophic, or hyperplastic prostate cells may be necessary to prepare chromatin for the subsequent molecular events for these processes (123).

Large differences were seen between chromatins isolated from myeloblastic and monoblastic leukemia patients. All the major histones were present in the monoblastic cells and lymphocytic cell extracts, whereas the loss of histones H1/ H3 and in some cases H2A and H2B was apparent in leukemias of myeloid origin. When lymphocyte chromatin was incubated with myeloblast chromatin, the lymphocyte histones were

degraded (124). This seems to indicate a potent protease present in granulocytes rather than a difference in composition.

A protease capable of hydrolyzing, in vivo, a liver catalase-depressing substance (toxohormone) into inactive peptides was present in the nuclear fraction from Rhodamine sarcomas of rats. Three proteases were found in the tumor with different proteolytic activity against histones and non-histone proteins. They were distinguishable from one another by their sensitivity to protease inhibitors (125).

In nuclei isolated from mouse ascite cell lymphoma KK/ly and from rat liver, protease activity is revealed mainly towards the arginine enriched histones E3 and H4. The proteolysis of the arginine enriched histones in tumor cell nuclei is more complete. Of various tissues tested, the tumor and brain non-histone acid-extractable proteins in the nuclei with low electrophoretic mobility also showed a high sensitivity to proteolysis (126).

8.0 Non-Histone Protein

Non-histone proteins (NHP) of the chromasomes are the proteins other than histories that are associated with chromati which like histones are synthesized outside the nucleus. Some are immediately associated with DNA but various periods of time elapse before others appear as components of the chromatin (1, 2) . The NHP exhibit a spectrum of stabilites with half lives that range from several minutes to several cell generations (3). As a group they exhibit a faster rate of turnover than the histones and are in a state of dynamic flux. NHP's exhibit a tremendous amount of heterogeneity structurally as well as functionally. Some NHPs appear to play a key role in stabilizing and maintaining the compact and folded structure or the DNA without histones (4). They range in molecular weight from under 10,000 daltons to over 150,000 daltons (5). Even within the same organism there are large differences between NHPs in different tissues; variation in the complement of NHP in the various cell types is consistent with the selective utilization of genetic information, AS opposed to histones, the amino acid composition of the NHP show an excess of dicarboxylic amino acids over the basic amino acids and are often referred to as nuclear acidic proteins (6). They show an enrichment in glutamic and aspartic acid (7).

Eukaryocytes show specific non-histone proteins (NHP) that associate selectively to DNA o£ the same species. In E Coli, there are specific binding proteins to DNA, however there are no histones (8). It has been suggested that the large groove of core particle DNA is a binding site for the NHP (9) and that the high mobility (HMG) basic NHP's interact with linker DNA in chromatin (10).

The NHP fraction is thought to contain proteins that have structural as well as regulatory roles in chromatin. The observed species specificity of NHP is consistent with a regulatory function (11, 12). One group of NHPs appears to endow pure DNA with nuclear acceptor sites (13).

8.1 Alterations of NHP in the Cell Cycle

Changes in the composition and metabolism of various molecular weight classes of NHP accompany modifications in ge: expression associated with development, differentiation (both in vivo and in vitro) , stimulation of cell proliferation, progression through the cell cycle, responses to steroid hormones and various pharmacological agents, viral infections and transformation, cellular aging and a broad range of other biological phenomena (14). In the differentiated state there is a selective decrease in the synthesis of NHP of high molecular weight that is reversed when the state of differentiation is reversed.

There is a strong correlation between the types of NHP associated with the genome and the alternation in gene expression and differences in RNA synthesis that accompany differentiation (15). The qualitative difference in NHP is seen in different stages and is exemplified by the increase in stimulatory activity in regenerating liver (16).

8.2 Enzyme Components of Non-Histone Proteins

One indication of their functional diversity is the various of complex enzyme systems found among them. Functionally they are defined as structural, enzymatic and regulatory macro molecules. There are enzymes such as polymerases involved in DNA and RNA synthesis and repair, enzymes involved in the processing and degradation of proteins and enzymes that modify nucleic acids and proteins by adding or removing acetate, methyl and phosphate groups. Chains of poly (adenosine diphosphate ribose) are found associated with certain NHP's, and glycoproteins as well as glycosaminoglycans have been show to be components of NHP (17).

The following list shows the variety of enzymes associated with NHPs.

Enzyme Components of the NHP (74)

Nucleic Acids as substrates DNA polymerases	**Polymerization of deoxy nucleotides into DNA**
RNA polymerases	Polymerization of ribonucleotides into processing or degrase RNA and/or DNA
Nucleases	
Nucleotide ligase	
Nucleotide exotransferases methylases	Joining of DNA segment
Chromosomal proteins as substrates Proteases	Processing or degrease proteins
Methylases	Methylation of histol
Kinases	NHP
Poly (adenosine diphosphate ribose) polymerase	Phosphorylation of and NHP
Poly (adenosine diphosphate ribose) glycohydrolase	Addition of ADP rib
Acetylasea	
Deacetylases	

8.3 NHP and Histones in Transcription

Histones and NHP both affect the base composition of RNA in transcription, and seem to work together. Nuclear transplant experiments show complete reprogramming from the restrictional specificity of the transplanted nucleus by substances in the cytoplasm (18). Adding NHP to a cell free system containing purified DNA and RNA polymerase could reverse the inhibition of RNA synthesis that is normally produced by the presence of histones. When a histone is complexed with NHP, the histone exerts only a partial inhibitory effect on RNA synthesis. The augmented transcription produced by the addition of NHP represents the activation of regions of the genome that had been previously suppressed. The NHPs dictate the elaboration of information from specific genes during defined periods of the cell cycle (19). Both histones and NHPs are synthesized in the cytoplasm. Hormones can affect the quantity and quality of NHP synthesized (20), and bind to specific histones in select target tissues. The binding of Cortisol is to an NHP (a phosphoprotein) and may

be a carrier protein that then binds to histones. Estrogen and hydrocortisone bind to specific receptor proteins in the cytoplasm and migrate to the nucleus. They affect target cells by binding in the nucleus and ultimately altering the pattern of gene transcription (21). Certain carcinogens alter the transcription of chromatin.

Followed by an induction on enzymes suggesting select gene derepression. One of the enzymes increasing its activity is DNA polymerase. Some carcinogens bind to histones and NHP, competing with hormones and are tissue specific. These carcinogens may also degranulate rough endoplasmic reticulum releasing ribosomes (22, 129-132).

In rat uteri, estrogen treatment of animals causes an increase in the labeling of NHP secondary to RNA synthesis (23 p. 140). This NHP may be a protease since estradiol and estradiol with progesterone induce a trypsin like protease in the ovariectoraized mouse (24) which is capable of degrading histones and other NHP (25). This may be part of a continuou chain of activities.

Chromatin templates devoid of histones showed alterations in the base composition of newly synthesized RNA and template for more species than any of the partially dehistonized samples. The sequence of nucleotides on the DNA which were previously covered by histones differed in their base ratios from those originally available for transcription (with histones). In addition to the histone effects on transcription, when NHP were eliminated and replaced with bovine serum albumin, and reconstituted with the DNA, the resulting nucleohistone although transcriptionally active, supported the in vitro biosynthesis of non-specific and randc RNA species (26, p. 144).

The administration of a steroid hormone ecdysone (molting hormone), to the larva of certain Dipteran species induced selected parts of their polytene chromosomes to become activated with respect to RNA and protein synthesis, similar hormones in vectabrate chromatin. The extended bands of RNA synthesis can be seen under light microscope as “puffs” on the chromosomes and correlate to the appearance of specific proteins whose biosynthesis was most likely coded by the RNA produced in these puffs (27). NHP can be found in these pufi and can cause tertiary folding of DNA (28). A DNA-protein complex has been proposed in which supercoiled DNA is folded into loops by several kinds of NHP (29).

It is quite possible that a reason for the difference in RNA base composition with histones and NHP is due to tertian folding which could either cause a reverse in transcription in E Coli (30, p. 294) e.g.:

	4 3
1 2 3 4 5 6	1 2 3 5 6
without histones and NHP	with histones and NHP
RNA = 1-2-3-4-5-6	RNA = 1-2-4-3-5-6

or that the NHP and histones allow a bypassing of codons in transcription.

3 5	
1 2 3 4 5 6	1 2 4 6
without histones and NHP	with histones and NHP
RNA = 1-2-3-4-5-6	RNA = 1-2-4-6

Additional alterations of NHP both qualitative and quantitative are seen in malignancies that cannot be accounted for by cell cycle dependent changes (31).

8.4 NHP Phosphorylation

NHPs are continually being modified in the nucleus by the addition and removal of phosphate groups, phosphorylation of NHPs is cell cycle dependent and associated with cellular differentiation and the activation of genes (32, 33). It is higher in the S-phase than during mitosis (34) and may be related to control of cell proliferation initiating DNA synthesis (35, 36). Changes in Histone H1-DNA-NHP interaction; occur in vivo, associated with phosphorylation of H1 molecules. The changes appear to be responsible for the relaxation of compact Gl phase chromatin superstructures into more open S-phase configuration during the Hela S-3 cell cycle (37).

Specific NHPs are phosphorylated at different ages in the life cycle, indicating different gene activation and repression at different phases of the life span. In old rats and young rats, phosphorylation of NHPs is higher than adults (38). The age dependent differences in phosphorylation of NHPs may be due to differences in protein kinases and phosphatases which control phosphorylation (39). Histones, on the other hand, show a decrease in phosphorylation and acetylation with age, but methylation is higher in the old than in the adult (cat). The NHPs show a decrease in acetylation and methylation with age (40). Alterations in protein-DNA binding occurs as a result of changes in the extent of acetylation, methylation and phosphorylation of NHP (41).

The phosphorylated NHPs are negatively charged and can repel the negatively charged DNA and also become strongly associated with the positively charged histones. The effect these two processes is to cause the displacement of the histo NHP complex from DNA (42).

Divalent cations such as Ca++ affect the catalytic activity of protein kinases (43). An increase in Mg++ affects the phosphorylation of Histone H2B by the cGMP dependent kinase (44). Some kinase fractions phosphorylate both histones and NHPs. Histones (in vitro) influence the ability of some fractions to phosphorylate particular NHPs and NHPs also appere to affect the in vitro phosphorylation of histones (45). Some

NHPs have been reported to be phosphorylated by cAMP independent protein kinase in the mouse spleen cell. This

phosphorylation is remarkably stimulated by double-stranded DNi but inhibited by whole histone or histone H2A (46).

Calcium stimulates phosphorylation of NHPs but can inhibit phosphorylation of histones. Calcium competes with histones for binding to DNA by displacing histones and interacting with the DNA phosphate groups (47). Lead competes with calcium and will bind to phosphates (48).

The ability of NHP to stimulate RNA synthesis in cell-free systems has been shown to be dependent on their state of phosphorylation (49).

Hormones can affect the phosphorylation of histones and NHP. In the rat cerebral cortex, estradiol stimulates phosphorylation of total histones and NHPs of adult and immature rats but has no significant effect in old age. Immature rats' histones and NHPs also show an increase in acetylation from estradiol, but methylation of chromosomal proteins was seen only in adult rats (50).

8.5 Phosphoproteins

A special class of NHPs located in the nuclei are the phosphoproteins which appear to activate RNA transcription. They have several phosphorylated serine or threonine residues per molecule. Kamiyama and Wang isolated a fraction of nucleal NHPs containing phosphoproteins and phosphokinase which activated RNA transcription in isolated chromatin. The phos-phoprotein fraction isolated from calf thymus nuclei contains a kinase enzyme which in the presence of ATP and Mg++ can phos-phorylate phosphoproteins in vitro, and a phosphotase enzyme capable of dephosphorylating the nuclear phosphoproteins (51, 52).

Teng et. al. pointed out the similarities between nuclear phosphoproteins in eukaryotic cells and the Sigma factors of bacterial RNA polymerases. The selective transcription of DNA genes in bacteria appears to be regulated through the recog-nition of individual gene initiation (promoter) sites by the Sigma factors of RNA polymerase molecules. Interaction betwee: these promoter sites and the Sigma factor leads to the initia-tion of transcription of the selected structural genes. After initiation, the Sigma factor is released from the transcribing enzyme and can interact with another molecule of RNA polymeras in a cascading action (53). E. Coli polymerase Sigma factor can be phosphorylated in vitro by animal phosphokinases. This process was stimulated by cAMP and the phosphorylated enzyme was more active in transcribing DNA from eukaryotic (calf thymus) cells than the controls (54). Cell Wall Deficient Bacteria have been found associated with cancer cells. Whethe this could be related to cancer etiology or merely circumstantial is being considered especially since various protein kinases are synthesized by bacteria (55), phosphorylation by kinase (s) is seen by certain RNA oncogenic viruses and may produce the malignant changes evoked by those viruses (56).

8.6 NHPs in Cancer

It is possible that intracellular bacteria or viruses may induce tumors in mammals by releasing proteins or enzymes that can activate transcription and cell division either directly or indirectly. The NHPs from viral mammary cancer cells are able to activate transcription of specific genes by eukaryotic RNA polymerase (57).

A specific antigen has been found in Novikoff hepatoma an Walker 256 sarcomas that appears to be a NHP not found in the chromatin of either normal or regenerating liver (58).

A protein kinase subfraction was observed in Novikoff hepatoma, Ehrlich ascites and Walker tumor nuclei that had the unique property of preferring Mn++ to Mg++ for optimal kinase activity, and showed a unique phosphoprotein as substrate (59).

Human lung carcinomas possess two distinct cationic proteins different from normal lung tissue (60). In Morris hepatoma, a decrease in certain basic proteins and an increase in some acidig non High Mobility Group Proteins was seen (61) in addition there was a decrease in the lysine rich El extract from hepatoma nuclei indicating either an increase in binding of histones to chromatin, or a decrease in amount of H1 available for chromatin (62).

The NHP: DNA ratios have been shown to be significantly increased in hepatomas and human leukemia cells (63). Montag et. Al. compared NHPs of rapidly and slowly

proliferating transplantable hepatocellular carcinoma (THC) with normal and regenerating rat liver and found a three-fold increase in the quantity of NHPs from the normal and regenerating rat liver (64). Ten NHPs were isolated in hepatocellular carcinoma that were absent from normal liver (65).

The NHPs in experimental rat hepatomas, mouse primitive teratocarcinoma cells, L-cells and neoplastic mammary cells C3H mice and Fischer rats also show qualitative and quanti-tative differences from their normal counterpart including proteins found in the cancers absent from normal tissue and vice versa (66).

In breast carcinoma besides the higher net synthesis of high molecular weight acidic proteins, there was a lower net rate of phosphorylation of these proteins (67).

The purified NHPs from mouse mammary cells bind specifi cally to homologous DNA or chromatin (68). The myeloma NHPs characterized by somewhat stronger binding to single strand repetitive DNA which could destabilize the natural DNA conformation. The repetitive DNA is thought to contain regulatory DNA sequences serving as binding sites for regula-tory molecules (69).

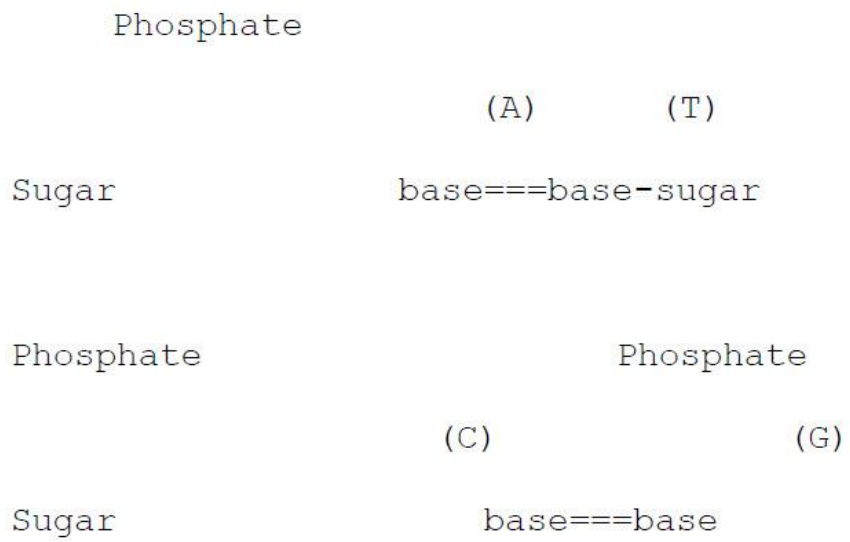
NHPs and phosphorylation of tumors show alteration after regression by hormone treatment. Examples of this are the reduction in nuclear volume of endometrial carcinoma cells responding to progesterone (70) and (P 1798) lymphosarcomas regressing after Cortisol treatment which showed a decrease in phosphorylation of the high mobility group proteins. No such inhibition of phosphorylation was seen in Cortisol resistant strains of this tumor (71).

Qualitative NHP differences were also seen with NHPs between proliferative and secretory endometrium and endometrial carcinoma (72). Finally, even NHPs in different tissues can be affected by tumors. When Rhodamine sarcoma grew on the back of rats, NHP of group II (40,000 - 100,000 M.W.) and group III (30,000 - 40,000 M.W.) in liver nuclei changed quantita¬tively, some increased while others decreased in content. The histone content, however, did not change. Some NHPs of group III in liver nuclei, the content of which decreased with tumor growth, were mainly phosphorylated when the nuclei were in¬cubated with ATP and the extent of phosphorylation decreased greatly with tumor growth (73).

9.0 DNA: RNA

RNA is transcribed from DNA. DNA and RNA are very similar in structure. RNA is a long unbranched molecule containing four types of nucleotides linked together by 3'-5' phosphodiester bonds. A nucleotide consists of a purine or pyrimidine, a sugar and a phosphate group, TWO differences ir its chemical groups distinguish RNA from DNA. The sugar of DNA is a deoxyribose, whereas RNA contains ribose, identical to deoxyribose except for the presence of an OH group. The second difference is that RNA contains no thymine but instead contain the closely related pyrmidine uracil. RNAs are single strand but can form double helical structures like DNA. They often fold back upon themselves in a hairpin configuration.

During DNA replication and during RNA synthesis the two strands of DNA must separate. The general structure of the nucleotide binding is shown below:



In DNA adenine binds with thymine and cytosine binds with guanine by hydrogen binding (1). Modifications of purine and pyrimidines can alter the complementary binding of bases. A low molecular weight RNA, and the RNA are specific for the attachment of individual proteins which can then be incorporated into a peptide or protein molecule in the riboson on RNA. Some of the RNAs are altered in transformed cells.

Nitrous acid is capable of deaminating adenine to hypoxanthine which pairs with cytosine instead of thymine (2). Spontaneous deamination of normal methylated bases such as 5'-methyl cytosine occurs in approximately 100 human cells per day causing a change from 5'-methyl cytosine to thymine which results in a change in pairing. Whereas dearaination of cytosine leads to uracil which is removed as part of the normal repair process, thymine from 5'-methyl cytosine is not removed and therefore left as a mutation form the original DNA code (3). During cell division the glycosylase repair enzymes are decreased and therefore an increase is spontaneous in mutation is possible thus leading to aberrant coding and in some cases tumorigenesis.

9.1 Methylation of DNA and RNA

Protection of DNA from foreign DNA such as viral or bacterial is possible with the combination of two enzymatic processes called modification and restriction. Modification: the enzymatic alteration of its own DNA by the cell in a species distinctive way, thus differentiating it from that of other species. Restriction is the action of specific enzymes

which can degrade foreign invading DNA's but do not destroy the host cell DNA, simply because the specific covalent alterations of the host cell DNA produced by the modification enzymes does not allow the restriction eynzyroes to act upon it. The protective modification of the host cell DNA is brought about by modification methylases which methylate certain adenine residues. Once the host-cell DNA is modified in the manner, it cannot be degraded by that cell's restriction enzymes. S-adenosyl-L-methionine (SAM) is the sole methyl donor for all mammalian transmethylases except those involved in the biosynthesis of methionine. L-methionine is converted into SAM by the enzyme ATP: L-metionine S-adenoysltransferase. SAM is also a precursor of the polyamines spermine and spermidine. An increased tRNA methylase activity which catalyzes the transfer of a methyl group from SAM to acceptor sites in tRNA has been found in several experimental and human tumors. Also a correlation appears to exist between the growth rate of tumors and the increased tRNA activity (4). The modification methylases transfer methyl groups from s-adenosyl- methionine to pairs of adenine residues in duplex DNA one in each strand; the two adenines are on adjancent or nearby base pairs. The sequence of bases on the two strands between and near the methylated adenines is symmetrical on either side of a midpoint. The methylated groups protect the host-cell DNA from enzymatic attack.

Modified host	3' C-T-T - A*-A-G-5'
DNA	G-A-A*- T-T-C-
Foreign Sequence	C-T-T - A-A-G
Attacked	G-A-A - T-T-C

(*methylated)

Some viral DNAs are modified for protection against host-cell restriction enzymes by transfer of glucosyl residues from UDP-glucose to hydroxymethylcytosine residues, In addition to protection of DNA, repression of DNA can occur with methylation of certain bases (5). Age and genetic factors are important in regards to methylating activities. Genetic factors appear to show predisposition of some strains within certain species for tumor incidences. The ability of a cell to remove or metabolized methylated purines correlates with its susceptibility to cancer by some agents (6). Changes in the pattern of DNA methylase activity as well as the decrease distortions in the character of DNA methylation may underlie the disturbances in the regulation of transcription of genome and cause the transformation of cells (7).

9.2 Methylation of DNA in Cancer

Alterations of DNA from carcinogens may not only be due to direct alkylation or damage by the carcinogen, but to activation of endogenous enzymes that can affect a

cell in such a way as to allow spontaneous transformation. One such possibility comes from the activation of methylating enzymes, and is affected by species differences in removal enzymes.

Guanine methylated at the O6 position (O6 methylguanine) causes the incorporation of non-complementary nucleotides during polynucleotide synthesis and may be one of the molecular basis of the mutagenic and carcinogenic effects of alkylating agents (8). The observation that almost all the mutations induced by N-methyl-N-nitro-N-nitroso guanidine and ethyl methane sulfonate are GC to AT transitions is in agreement with this suggestion.

O6 methylguanine is produced in greater yield or persists for longer periods of time in the DNA of the principal organs in which tumors arise than in the DNA of organs in which tumor; do not occur. It is removed more slowly from the colon than from other organs (9). The active removal of O6 methyl-guanine from DNA in vivo appears to be due to an enzymatic mechanism which varies from tissue to tissue and is lowest in the organs most susceptible to tumor induction.

Low level exposure to dimethylnitrosamine actually increases resistance to mutagenesis by activating removal enzymes for O6 methylguanine but high doses overcome the capacity of the induced enzymes to repair DNA thus increasing the risk of cancer in animals receiving large doses.

Continuous exposure to low doses of DMN results in a lower amount of O6 methylguanine in liver DNA of exposed rats than in liver DNA of rats not exposed. This seems to indicate an increased rate of removal in exposed rats which incidentally is specific for O6 and not 3-methyl or 1 methyl guanine (10).

In mice with a low susceptibility to dimethyl hydrazine-induced colon carcinogenesis (C57 BL/H), concentrations of 7-methyl guanine and O6 methyl guanine in DNA of colon ileum and kidney were 40 to 60% less than in mice with a high incidence of colonic tumors (ICR/H). In all organs investigated, the metabolic incorporation of C14 into normal DNA bases was lower in C57 BL/H than ICR/H mice. The low carcinogenic response of C57 EL/H mice may be due to the smaller extent of initial alkylation of colon DNA reflecting differences in the enzymatic metabolism of the parent carcinogen. The high susceptibility of the ICR/H strain follows Mendelian law, being dominant (11).

Methylation of DNA covers a wide range in regards to cancer cells. The methylation level for all the DNAs from ascite leukosis cells (L 1210) and hamster embryonic cells transformed by virus SV-40 and polyoma was higher than for normal mouse and rat liver DNA (12).

In vitro, DNA methylase activity found in the nuclear extracts of leukemic lymphocytes methylate the cytosine residues of DNA in other sequences than enzyme(s) of the hypertrophic, or hyperplastic prostate cells may be necessary to prepare chromatin for the subsequent molecular events for these processes.

Large differences were seen between chromatins isolated from myeloblastic and monoblastic leukemia patients. All the major histones were present in the monoblastic cells and lymphocytic cell extracts, whereas the loss of histones H1, H3 and in some cases H2A and H2B was apparent in leukemias of myeloid origin. When lymphocyte chromatin was incubated with myeloblast chromatin, the lymphocyte histones were degraded. This seems to indicate a potent protease present in granulocytes rather than a difference in composition.

A protease capable of hydrolyzing, in vivo, a liver catalase-depressing substance (toxohormone) into inactive peptides was present in the nuclear fraction from Rhodamine sarcomas of rats. Three proteases were found in the tumor with different

proteolytic activity against histones and non-histone proteins. They were distinguishable from one another by their sensitivity to protease inhibitors.

In nuclei isolated from mouse ascite cell lymphoma NK/ly and from rat liver, protease activity is revealed mainly towards the arginine enriched histones H3 and H4. The proteolysis of the arginine enriched histones in tumor cell nuclei is more complete. Of various tissues tested, the tumor and brain non-histone acid-extractable proteins in the nuclei with low electrophoretic mobility also showed a high sensitivity to proteolysis.

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Chapter One
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Chapter 3
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Chapter 4
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Chapter 5
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Chapter 6
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Chapter 7
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Chapter 8
References -- Non-histone Proteins

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Chapter 9
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About the Author

Dr. Dana Flavin

Born in Chicago as the youngest child of Dr. and Mrs. Byron Flavin. Both parents studied Medicine and their excellent example of compassion and devotion to duty made a lasting impression on Dr. Flavin's life. Her continuous education in Science and Medicine has helped her to find and improve therapies for many diseases including Cancer, Autoimmune diseases such as Graves thyroiditis, rheumatoid arthritis and viral diseases. She received her first degree in Psychology and Chemistry from Loyola University in 1972 and then began graduate school in Pharmacology at Chicago Medical School. After 4 years and part I of her Medical Boards, she was appointed Science Assistant to the Associate Bureau Director for Toxicology at the Food and Drug Administration in Washington D.C. . During this time she researched the Molecular Biology of Cancer and Tumor Promotion investigating the application of Translational Medicine into potential therapies for cancer. She then was appointed Science Advisor to the President of the Nutrition Foundation and began graduate studies at Howard University in Nutrient Biochemistry under the Department of Nutrition. Several years later with summa cum Laude in Nutrition she moved to Germany and began her studies in Medicine for completion of her M.D. degree in Innsbruck, Austria, including years devoted to research.

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