CELLULAR EFFECTS OF ANTICANCER DRUGS[1,2,3]

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INTRODUCTION

Pharmacology is involved in a broad new research area—that concerned with the effects of drugs on proliferating cells. The work in this field has extended rapidly because of urgent public pressure for effective drugs against cancer and recent major discoveries in cellular biology. As the first contribution to the *Annual Review of Pharmacology* on this subject it is appropriate to review some general principles related to the pharmacology of growth-inhibiting drugs.

A review of anticancer drugs would be expected to discuss those drugs which destroy or inhibit the growth of neoplastic cells without undue injury to the normal tissues of the host. An effective anticancer drug should have a specific action on a vulnerable system unique to the cancer cell; the presence of such a unique metabolic pathway might even be the cause for the neoplastic properties of the cell. Unfortunately, neither a common cause nor a unique metabolic disturbance in cancer is known, and drugs with specific

[1] The survey of the literature pertaining to this review was concluded in October, 1962, with a few exceptions.

[2] Abbreviations used in this chapter include: BU (bromouracil); BUdR (5-bromo-2′-deoxyuridine); CdRTP (2′-deoxycytidine triphosphate); CU (chloro uracil); CTP (cytidine triphosphate); FC (5-fluorocytosine); FCdR (5-fluoro-2′-deoxycytidine); FCdRTP (5-fluoro-2′-deoxycytidine triphosphate); FGAR (formylglycineamide); FO (5-fluoroorotic acid); FOMP (5-fluoroorotidine 5′monophosphate); FU (5-fluorouracil); FURMP (5-fluorouridine 5′monophosphate); FUDRP (5-fluorouridine diphosphate); FURTP (5-fluorouridine triphosphate); GDH (glutamic dehydrogenase); GMP (guanosine monophosphate); GTP (guanosine triphosphate); HAD #1 (human adenocarcinoma #1); I-B-C UdR (5′ iodo bromo and chloro 2′-deoxyuridine); IMP-GMP (inosine monophosphate-guanosine monophosphate); IU (5-iodouracil); IUdR (5-iodo-2′-deoxyuridine); T (thymine); TdR (thymidine); 6AzU (6-azauracil); 6AzUR (6-azauridine).

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anticancer activity have not been found. Drugs presently used to inhibit neoplastic cells also appear to act on similar if not identical metabolic pathways present in normal cells, and while certain drugs do exhibit consistent therapeutic activity against some forms of cancer in animals and man, they are generally administered in near-toxic doses in order to achieve a response. A thorough understanding of the mechanism of action of these drugs on various types of normal and neoplastic cells in several species and when administered in different ways may increase the ability to use them against human cancer to their maximum therapeutic advantage.

Levels of Pharmacological Analysis.—The growth-inhibitory drugs have been investigated in biological systems of varying complexity (1, 2). These include studies with these agents (*a*) at the molecular level—reactions with certain intracellular substances and effects on enzymatic functions; (*b*) at the cellular level—effects on cellular structures and metabolic activities; (*c*) in various species, including viruses and other micro-organisms, plants, invertebrates and vertebrates; (*d*) on developing embryonic systems, including a search for teratogenic activity; (*e*) on regenerating structures; (*f*) on organ functions, such as the bone marrow, liver, digestive tract; (*g*) on neoplasms spontaneous and transplanted, in several species; (*h*) for carcinogenic activity; (*i*) for mutagenic effects; (*j*) for distribution and metabolism in animals including man; (*k*) on mechanisms of resistance in whole animals and in isolated systems; (*l*) on the value of protective chemicals, *in vitro* and *in vivo;* (*m*) on enhancing their activity in combination with other drugs or radiation; (*o*) on their administration by various routes including oral, intravenous injection, prolonged intravenous infusion, local perfusion and intra-arterial infusion. The structural analogues of active drugs are examined for enhanced biochemical activity and for specific effects on certain types of normal and neoplastic cells. Finally, the influence of a drug on a cancer must be interpreted, taking into consideration its direct effect on the cancer cell, on the blood supply to the cancer, on the connective tissue proliferation stimulated by the tumor, and on the immune mechanism of the host (3).

Studies at each level must be separate, and data from one system are not necessarily transferable to another. Since it is not possible in this review to discuss the major growth-inhibiting drugs at their several levels of action, we propose to examine their effects at the cellular and molecular levels. Subsequent reviews may consider these drugs from other points of view. The physiological disposition of anticancer drugs was recently reviewed by Mandel. (4)

Methodology.—Recent advances in our knowledge of cellular function and the action of drugs on cells have stemmed largely from technical developments, which have permitted studies at a subcellular and molecular level. These methods include: (*a*) electron microscopy (5); (*b*) localization within the cell of precursors labelled with tritium, by radioautography of electron microscope photographs at a very high level of resolution (6) and in the light microscope (7); and, (*c*) cell fractionation methods for chemical and enzymatic studies on cell components, including centrifugation and chroma-

tography, with and without the use of labelled precursors. These methods are applied in association with commonly used strains of bacteriophage and other viruses, bacteria, normal and neoplastic mammalian cells in tissue culture and *in vivo*, and tissue extracts and cell fractions.

CELL STRUCTURE

Before discussing the effects of growth-inhibiting drugs on the cell, it is necessary to appreciate some of the recent data on cell structure and function and the mitotic cycle. A hypothetical model cell will be described based on information from various sources, but quantitative and qualitative differences undoubtedly occur in different systems and organisms. Figures 1 and 2 show some of the operational principles diagrammatically, and within these frameworks the proposed sites of action of various drugs will be discussed later. Since most growth-inhibiting drugs act on sequences concerned with nucleic acid synthesis and metabolism, emphasis will be given to these components of the cell.

Nucleus.—The cell nucleus is bounded by a double-layered nuclear membrane with pores or annuli in the outer layer through which it is thought that large molecules may communicate between the cytoplasm and nucleus (8). The nucleus is made up of chromatin, one or more nucleoli, and an amorphous proteinaceous matrix. DNA is found exclusively in the chromatin, and in resting or non-dividing cells the chromatin is diffusely distributed throughout the nucleus as long filamentous strands, presumably to allow maximum surface area for contact with RNA and other material. RNA is distributed throughout the nucleus, but it is particularly concentrated in the nucleolus. Both DNA and RNA are polymers composed of nucleotides. Each nucleotide is composed of one of the purine or pyrimidine bases linked to a 5 carbon sugar which is phosphorylated in the 5′ position. RNA contains the ribose and DNA the deoxyribose sugars, but in both the nucleotides are linked by phosphodiester bonds between the 3′ and 5′ carbons of the sugars (9 to 11). The biosynthetic pathways of the purine and pyrimidine bases and the pentoses have been reviewed in detail previously (12 to 14).

With rare exceptions, the structure of DNA is that of a double helix formed by two strands of polymerized nucleotides which are linked together by hydrogen bonds between the bases of the two chains (15). Adenine (A) is paired with thymine (T) (AT) and guanine (G) with cytosine (C) (or 5′-methylcytosine) (GC) (16). The molecular weight of DNA is uncertain because of physicochemical problems in isolating and purifying the intact molecules as they occur in the nucleus, however, it is estimated to be in the order of 6 to 10 million (17). Each strand of DNA is complementary with the other and each provides a template for the *de novo* synthesis of its complementary mate when replicating preparatory to cell division. At cell division, each daughter cell receives one of the original strands of DNA and one which is newly synthesized (18 to 20). The genetic code of the cell is determined by the sequence arrangements of the four bases in its DNA (21, 22).

RNA is also composed of bases, which differ from those of DNA in that

uracil is present in place of thymine. There are several different types of RNA, and this has not been conducive to establishing a reliable structure of the molecule.

Based on studies in bacteria, it has been proposed that "messenger" RNA (mRNA) acts as an intermediary in the transfer of genetic information from the nuclear DNA to ribosomes, where specific proteins are formed (23). Recent advances have been made in studying newly synthesized RNA in *E. coli* and other bacteria infected with a DNA phage; this RNA has a base sequence complementary to the phage DNA and appears to function as an mRNA (24 to 28).

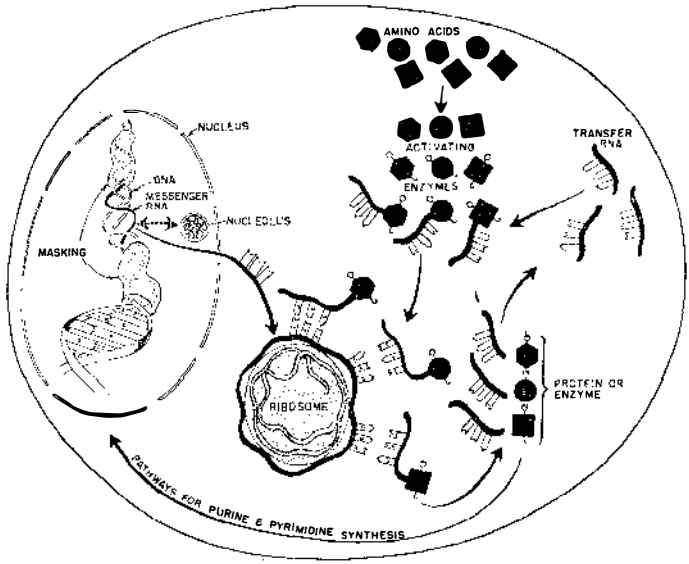


FIG. 1. Diagramatic concept of factors involved in controlling synthesis of specific proteins. mRNA is formed through the action of an RNA polymerase on a "structural" component of the DNA which has a complementary base sequence. The role of histones and other chromosomal proteins in "masking" portions of the DNA not used in templating mRNA, the mode of action of "regulatory" genes and repressor and inducer substances that govern the rate of mRNA synthesis, and the function of the nucleolus are not clear at the present. The mRNA passes into the cytoplasm where in some manner it attaches to a ribosome to direct specific protein synthesis. The function of the pre-existant "workshop" ribosomal RNA and its relationship with mRNA are not known. Amino acids are activated by specific enzymes and transported to the ribosomes by specific tRNA molecules. These specific tRNA molecules are aligned at particular sites on the mRNA through possession of a complementary base sequence, and thereby assure transcription of the proper peptide bonding necessary for assembly of the amino acids into specific enzymes. These enzymes act on specific substrates along defined biosynthetic pathways to produce the more complex materials necessary for cell function and reproduction.

The physical form of DNA when it serves as a template for RNA is not known. One theory favors the retention of the double helical form of DNA and the formation of a three chain intermediate (29). This is constructed as a triple helical hybrid complex so that its structural figuration may correspond to DNA and permit the necessary hydrogen bonding. A particular RNA polymerizing enzyme is necessary for polymerization of the mononucleotides on the DNA surface to form an mRNA. Thus, what type of mRNA molecule is formed may be under the joint control of DNA and RNA polymerizing enzyme. Current evidence suggests that mRNA is formed exclusively on a DNA template (30 to 36).

RNA is also contained in the nucleolus, but this has not been identified as to function; it appears to be derived from the chromosomes (37) possibly arising from the same mechanisms involved in mRNA formation.

mRNA probably has a molecular weight of 250,000 to 500,000 being made up to about 750 to 1500 nucleotides. Once formed on the DNA, bacterial mRNA is detached and "associates in the cytoplasm with pre-existing non-specialized ribosomal particles" (38). mRNA then directs the alignment of activated amino acids to synthesize specific enzymes. It is not known whether mRNA is destroyed after each transcription of a single protein (38) or

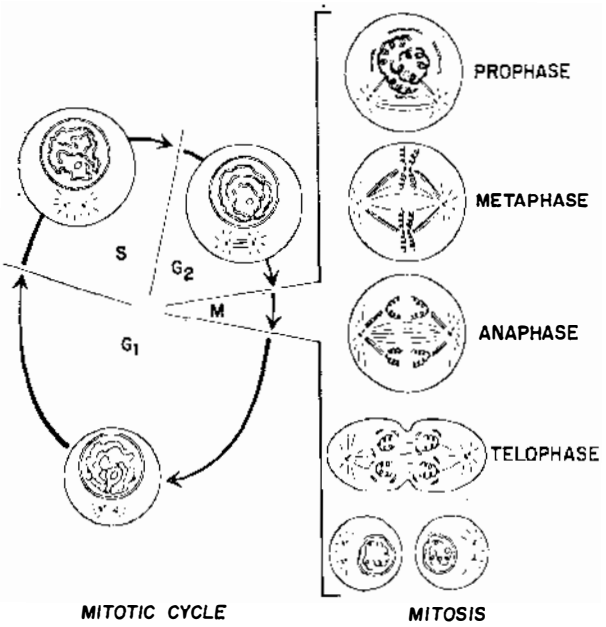


FIG. 2. Diagramatic representation of major events taking place during the different phases of the division cycle:

G 1—post-mitotic, S—DNA synthesis, G 2—pre-mitotic, and M—mitotic phases. These events are described in the text.

whether it can serve as a template for several repeated transcriptions of the same protein (39). Since one of the properties of bacterial mRNA is rapid renewal, longer surviving mRNA may be referred to in this context as "informational" RNA. In mammalian cells, the renewal rate of "informational" RNA may be much slower than in bacteria, and may function in a manner different from mRNA. It has been shown *in vitro* that part of a synthetic mRNA may be degraded rapidly, prior to and dissociated from protein synthesis, and the remaining portion of the mRNA which attaches to the ribosome may be able repeatedly to direct the formation of a specific protein molecule (40). It is generally thought, however, that bacterial mRNA has a short halflife, and that it is continuously being formed on the chromosomes and transmits a more or less continuous flow of information to the ribosomes for specific protein synthesis (41 to 43). The histones which are intimately associated with DNA, have been suggested as regulatory factors in mRNA synthesis (44). The portion of the DNA not complexed with histones may serve as the site for mRNA synthesis.

Cytoplasm.—The cytoplasm, when forming protein for export in secretory cells, contains a highly organized membrane system named the endoplasmic reticulum, as seen under the electron microscope (45). This membrane system furnishes a vast network of canaliculi through which intracellular fluids may circulate. Small ribosomal granules, composed of about 40 to 50 per cent RNA and the remainder protein, are attached to the surface of the endoplasmic reticulum, but some appear to be free in the cytoplasm. The Golgi apparatus appears to be in continuity with the endoplasmic reticulum, and may participate in its formation (46, 47). When the cell is disrupted and its contents homogenized and separated by differential centrifugation, the ribosomes are separated in the microsomal fraction (48). The ribosomes are the major site of protein synthesis in the cell (49 to 52). The relation of mRNA to ribosomal (rRNA) is not clear. Ribosomes apparently only become functional with the addition of mRNA. When *E. coli* ribosomes are studied *in vitro*, 4 per cent of the total RNA is mRNA, and only 10 per cent of the ribosomes have mRNA attached to them. The mRNA ribosomes sediment faster than the "free" ribosomes and only the "heavy" ribosomes are active in protein synthesis (53).

Protein synthesis.—Free amino acids enter the cell by transport across the cell membrane (54), or are synthesized in the cell by amination of α-ketoglutarate to form glutamic acid (55). The latter can undergo transamination to form any of the other non-essential amino acids (56). Each amino acid has a specific "activating" enzyme that prepares it for peptide linkage to another amino acid, with adenosine triphosphate (ATP) as an energy source. ATP is cleaved, releasing two of its three phosphates, and the resultant adenylic acid (AMP), forms a high energy complex with the carboxy group of the amino acid. The amino acid—AMP complex is bound to its specific enzyme (51, 52). The activated amino acid is transferred to a specific soluble "adapter" or "transfer" RNA (tRNA) which somewhere contains a sequence

of nucleotides characteristic for each amino acid. tRNA is of low molecular weight (27 to 30,000 with about 85 nucleotides), and has been referred to as "soluble" RNA in chemical procedures used in extracting nucleic acids (57).

Amino acid "transfer RNA" complexes are then transferred to an mRNA coded ribosome where the amino acids are linked together by peptide bonds to form specific proteins. The mRNA has consecutive nucleotide sequences, each complementary to at least a portion of a specific tRNA. The arrangement of tRNA molecules in relation to the mRNA nucleotide sequence lines up the amino acid in the sequence of a specific protein. The formation of the peptide bonds is a complex reaction, requiring the co-factor guanosine triphosphate (GTP), one or more soluble transfer enzymes, magnesium (58), and in some circumstances sulfhydryl compounds (59). The protein is then stripped off the ribosome and released into the cell. The biological properties of the cell are determined by the amino acid sequences of its proteins (60). Relevant to this postulated course of events is the demonstration in *E. coli* that the enzyme tryptophane synthetase is under the control of an identifiable gene; mutations in this gene result in the appearance of mutant proteins (61, 62).

The amino acid code of mRNA is now under intensive investigation. A "doublet" code has been suggested (51) although most workers favor a "triplet" code with three nucleotides in sequence being necessary for each amino acid. The "triplet" code contains the smallest number of nucleotide groups which allows sufficient different permutations to include a separate unit for each of the 20 amino acids, assuming cell coding units have three nucleotides (63–71). It seems likely that the coding scheme will be universal (60, 68, 72). Complicating the solution of the code are the possibilities that (*a*) some of the coding nucleotide configurations may overlap or not be contiguous, (*b*) more than one coding unit may correspond to a single amino acid ("degenerate" code), and (*c*) certain nucleotide sequences do not code any known amino acid ("nonsense" code). Furthermore, if the postulated mRNA codes for the known amino acids are correct, they result in an unnatural excess of uracil in the mRNA (73, 74). This discrepancy could be resolved if it could be shown that the code is "degenerate" with several symbols for a single amino acid that contain uracil, or that only a portion of the mRNA contains the structural amino acid code, while the remainder serves to regulate such functions as the rate and timing of specific protein synthesis (23, 60, 75). The term "informational RNA" has been used to include both messenger and regulatory RNA (24).

Mitochondria.—Electron microscopy has clearly demonstrated the mitochondria as a discrete double-walled structure (76). The inner membrane is involuted to form cristae or folds which enclose a soluble matrix. The membranes are made up of alternating layers of protein and lipid molecules (77). The main function of the mitochondria is to extract energy from the nutrients that are supplied to the cell, transforming this energy into the high energy phosphate bonds of ATP. It is thought that the Krebs cycle enzymes are

located in the soluble matrix of the mitochondria while the "respiratory chain" enzymes which include diphosphopyridine nucleotide (DPN), flavoprotein, and several iron-containing enzymes (the cytochromes), are located in the membrane as part of a monolayer of protein molecules (77). In the passage of the excited electrons down the respiratory chain, the electrons gradually give up their energy to drive the synthesis of ATP from ADP+phosphate (oxidative phosphorylation) (78). Sandwiched between the protein monolayer is a double layer of phospholipids that apparently serves to hold together and properly orient the groups of enzymes.

Lysosomes.—The lysosomes are cytoplasmic structures about the same size as the mitochondria which contain all of the acid hydrolytic enzymes of the cell, including cathepsins, acid phosphatase, β-glucuronidase, ribonuclease, and other hydrolases (79, 80). These enzymes are necessary for the degradation of large molecules into smaller components which can then be used either to synthesize required cellular constituents or to serve as a source of energy transformation by the mitochondria. The hydrolytic enzymes are thought to be encapsulated by a lysosomal membrane to protect the rest of the cytoplasmic components. If the lysosome ruptures because of environmental changes or toxic agents, hydrolases are released and cell autolysis occurs (81, 82). It has been proposed that physiological autolysis by lysosomal enzymes is in part responsible for the degeneration of certain embryonic tissues during development as well as in adult life (83). Release of lysosomal enzymes also have been related to inflammatory reactions and shock following endotoxin injection (84).

Centrioles.—The centrioles or mitotic centers are seen as "granules of extreme minuteness", or may be invisible in cells by light microscopy (85). The electron microscope, however, shows the centriole to be composed of cylindrical bodies about 0.3 to 0.5 microns long and 0.15 microns in diameter (86, 87). The cylinders are formed by nine groups of tubules arranged in cylindrical array, each group containing three tubules. The fine structure of the centrioles is quite similar to that of the basal granules or kinetosomes of cilia and flagella (88, 89). In some neuro-epithelial cells of the chick embryo, the centrioles have been traced to a position near the cell surface where they have become basal granules and form cilia (90). The role of the centriole in cell division is discussed under mitosis. The chemical composition of the centriole is not known (91).

Cell membrane.—The cell membrane is an elaborate structure about 75 to 100 Ångstroms in thickness, and it appears to be composed of a double layer of lipid molecules between two single protein layers. A layer of mucoprotein coats the exterior surface of most cells. There is a continuous exchange of substances between the cell and its environment. One mechanism for this is the active transport of substances; for example, even simple ions, notably sodium and potassium, show considerable difference in their concentration between the extracellular fluid and within the cell, with the intracellular concentration of potassium being about 40 times that in the environment. An-

other mechanism is referred to as pinocytosis wherein during the process of engulfing material from the environment that has been adsorbed to the cell surface, a portion of the cell membrane is recessed within the cell (92, 93, 94). The recessed area is pinched off from the cell surface to form a vacuole or vesicle ("phagosome") which migrates to the interior of the cell (95). Glucose (96), amino acids, and certain proteins (97) including insulin (95) and RNAse (98 to 100) may be pinocytosed by the cell. Some workers believe that lysosomes fuse with, and release their enzymes into, ingested vacuoles to aid in digesting the latter's contents (79, 101, 102).

MITOTIC CYCLE

The events leading to cell division and the act of division are a distinct and separate phase in the life of a cell. While some non-dividing cells are susceptible to certain toxic drugs, the complex events in the mitotic cycle create special vulnerable situations in which the "mitotic inhibitors" can disrupt the cell. Some recent information on mitosis is helpful in interpreting the site of action of these drugs. This subject has been presented in detail in a review by Mazia (91).

The mitotic cycle of higher cells can be divided into several phases as defined by the time relation between the incorporation of DNA precursors labelled with radioisotopes, and the appearance of labelled cells in mitosis (103 to 112). This is shown schematically in Fig. 2. The relative proportion of time spent in the various phases of the division cycle has been similar in the cell types studied thus far (110, 111). During the period between mitoses the cell is in *interphase*, and this has been divided into three intervals in relation to the synthesis of DNA preparatory to cell division.

G1 (post-mitotic interval) is the interval following mitosis and before the beginning of DNA synthesis, and usually comprises over half the total generation time. S is the period of DNA synthesis; this comprises about one fourth to one third of the generation time. G2 (pre-mitotic interval) is the period between the end of DNA synthesis and the beginning of mitosis, and comprises less than one fifth of the generation time. M is the period of mitosis and usually lasts about an hour. The generation time is the time it takes for a cell to complete one entire division cycle. It is an oversimplication to assign each of the biochemical events which take place in a cell in preparation for mitosis specifically to one of the particular phases of the division cycle. General metabolic processes necessary for maintenance of cell life, as well as RNA and protein synthesis and cell growth, go on throughout interphase. It seems probable, however, that the major part of each of the particular events to be discussed which lead up to mitosis occurs during the phase under which it will be described. It will not be possible here to discuss all the changes that take place during cell division, such as those involving the mitochondria 113) and endoplasmic reticulum (91, 114).

G1 (post-mitotic phase).—The chromosomes occur as filaments diffusely distributed throughout the nucleus, the nucleoli are present, the nuclear

membrane is intact, and in most animal cells, the two parent centrioles have already reproduced during the preceding division (91). In a number of different cells it has been shown both *in vitro* (109) and *in vivo* (107 to 120) that the variations in inter-mitotic time are usually due to a variation in G1 as opposed to other intervals in the mitotic cycle. In fact, the decision of a cell to divide generally appears to take place during the G1 phase (107, 121, 122), if it should fully differentiate instead and no longer be capable of division, G1 in this sense could be said to be indefinitely prolonged. Most cellular growth occurs during G1.

S (DNA synthesis phase).—During this period, the DNA content of the nucleus is exactly doubled (91, 121). The changes that take place in the physical state of the DNA molecule during replication are not entirely clear, but probably include unwinding of the double strands and splitting of the molecule in order to allow each complementary strand to be replicated separately (121).

In order for DNA polymerase to function in linking together the deoxynucleotides
DNA be present to act as a primer as well as a template for the formation of new DNA (123). In some systems, the native DNA appears to function as a primer whereas in others, denatured DNA is required. This suggests that DNAase has a physiological role in converting native DNA into a primer (124 to 127); however, this fact remains to be proved.

DNA exists in the cell as a nucleoprotein, and it has been shown that histone and other "chromosomal proteins" are synthesized more or less simultaneously with DNA (121, 128 to 131). The biosynthesis of histone and probably other proteins appears to be required for formation of new DNA, possibly serving to link DNA molecules together (132 to 135). RNA synthesis is depressed during DNA replication, but does not stop completely, probably because all the DNA does not replicate simultaneously and some fraction is always capable of templating RNA (136 to 139). Some of the factors that are known to influence DNA biosynthesis have recently been reviewed by Lark (121).

G2 (pre-mitotic phase).—During the pre-mitotic period, fine filaments appear in the vicinity of the centrioles that may signify beginning synthesis of proteins concerned with spindle formation (91). "Energy reservoirs" are also formed during this period, presumably involving accumulation of ATP in order to carry the cells through the mitotic process (91, 129). Inhibitors of respiration and oxidative phosphorylation will prevent mitosis if imposed prior to chromosomal prophase; but once division has begun, it continues to completion (129 to 143).

Although the variation in inter-mitotic time is usually due to variation in the length of G1, it has been shown that some epidermal cells may remain in "antephase" or G2 for long periods, and then shortly following injury by cutting of tissue, undergo mitosis (140, 144, 145). It is postulated that this population of cells, which have already doubled their DNA, but are holding

in G2, may be able to act promptly to repair tissues which normally have a slow renewal rate. Not only are these cells holding in G2 stimulated by injury to enter mitosis, but other cells, in the G1 phase, begin to synthesize DNA within an hour after the stimulus of injury, and subsequently divide (145).

M (mitotic phase).—The mitotic phase classically has been divided into four sub-phases: (*a*) prophase, (*b*) metaphase, (*c*) anaphase, (*d*) telophase.

Prophase: The most obvious event, recognizable in the light microscope, is the condensation of the chromosomes during which time they become tightly coiled and shorten in length (91, 140, 146, 147). The chromosomes split into two sister chromatids which may enter the prophase wound around each other. Each sister chromatid receives half of the old and half of the new DNA (148).

RNA synthesis appears to continue in the nucleus and nucleolus for a short time in the prophase, but then stops completely about mid-prophase. This presumably is because the DNA is, at this time, tightly compressed into "mitotic chromosomes" and is no longer able to serve as a template for RNA synthesis (30, 31, 36, 149 to 151).

Shortly after cessation of RNA synthesis in mid-prophase, the nucleolus breaks down and the nuclear membrane undergoes dissolution (36, 149). At the time of nuclear membrane breakdown, nuclear RNA is released into the cytoplasm (36, 149, 152, 158).

Protein synthesis, as estimated by the rate of incorporation of H^3-histidine, also begins to decrease significantly by mid-prophase (36). This decline continues until telophase, at which point it has fallen to 25 per cent of the average inter-phase level.

Other workers have observed an accumulation of RNA (153, 154) and of phospholipid (155), on the chromosomes during prophase. These substances are carried by the chromosomes through mitosis but are then released before entering telophase. The significance of these observations is not clear, but they may indicate that the chromosomes distribute these materials as well as DNA to the daughter nuclei.

Although the time of separation of the centrioles varies in different species, in many animal cells the mitotic apparatus begins to form during prophase and the centrioles start to move apart to establish the mitotic poles. The mechanisms by which the poles separate are not well understood but may involve a repulsion by which the elongating "continuous fibers" of the central spindle (between the centrioles) force the centers apart (91).

The "chromosomal fibers" connect the centromeres or kinetochores of the chromosomes to the poles. Most chromosomes are usually monocentric, i.e., have only one kinetochore (156). The structure and function of the kinetochores, and their role in chromosomal duplication and separation, are complex and not entirely understood, but they appear to be essential for mitosis (91, 157, 158).

Metaphase: At the end of prophase, the chromosomes move to the cell

equator to become aligned midway between the poles with each sister chromosome attached to a different pole. The fibers connecting the kinetochores of the chromosomes to the poles, those between the two poles in the central spindle, and those fibers growing from the centrioles toward the cell surface (astral rays) lead to the well-known metaphase picture. All these fibers have been shown by the electron microscope to be similarly composed of "spindle filaments" which are tubular structures tending to occur in pairs in association with fibrous elements (45, 159).

The mitotic apparatus has been defined by Mazia & Dan (160) to include the spindles, asters, centrioles, nuclei before breakdown, and chromosomal structures after breakdown of the nuclear membrane. It contains at least 10 per cent of the cell's protein (161), and possibly a much greater percentage (162). A few proteins make up most of its mass; these are rich in thiol (SH) groups which are capable of being oxidized to disulfide (S-S) bonds (161). When assembled, the mitotic apparatus is a gel created by association of protein macromolecules, probably involving intermolecular S-S and possibly other sulfur-containing links (91, 163 to 166). It has been proposed that hydrogen bonds serve to condense and orient macromolecular polymers into fibrous bundles (91, 167, 168).

The mitotic apparatus also contains RNA (169), lipids (91), ATPase (170), and zinc (91). The RNA could have to do with the assembly rather than the synthesis of the apparatus, as there is evidence against protein synthesis occurring during metaphase (36).

Anaphase: During anaphase, the essential feature is the separation of the sister chromatids. This takes place as a result of two movements: (*a*) a shortening of the chromosomal fibers which connect the kinetochore of each sister chromatid to one of poles, thus "pulling" them apart, and (*b*) an elongation of the fibers of the central spindle, thus expanding the "interzonal" distance between the poles and tending to "push" the chromosomes apart (91, 171 to 173). The asters undergo considerable enlargement and their rays may extend to reach the cell's surface (174).

Towards the end of anaphase, there is an apparent release of RNA that accumulated on the chromosomes during prophase, thus completing the "chromosomal RNA cycle" (91, 114, 153, 175, 176).

Telophase: During telophase vesicular elements possibly originating from the endoplasmic reticulum, collect on the surface of individual chromosomes where they fuse to form double membranes about each chromosome. As the chromosomes approach their pole, these separate membranes coalesce, and a continuous nuclear membrane is thereby reconstructed about the whole group of chromosomes (114, 177 to 179). The chromosomes then uncoil and re-assume their filamentous interphase configuration (91, 180). The nucleoli reform (181 to 184), the spindle and asters break down (91), and it is usually during this stage that each centriole produces a daughter centriole.

Cytoplasmic cleavage or cytokinesis may be independent of nuclear mitosis in some cells, such as striated muscle where telophase is not followed

by cytoplasmic division. In most cells, however, cytokinesis is usually associated with nuclear division and in animal cells occurs by "furrowing" with the plane of division determined by the axis of the poles (185, 186). The poles are indispensible (91) for cytokinesis, but the chromosomes (187) and spindle (188 to 191) do not appear to be essential. Several possible mechanisms of cell cleavage have been proposed involving contraction of the cell cortex at the site of furrow formation (192 to 194) and the participation of the asters and spindles (195); however, the actual manner of formation of new plasma membrane surface at the furrow that seals off the two daughter cells from each other is still unsettled.

CELL FUNCTION

The various cellular components with their inherent properties are adapting continuously to a changing environment, as well as responding to regulatory mechanisms essential for the orderly development and integration of functions in a multicellular organism. Each cell appears to respond in a characteristic manner within the limits imposed by its type to its environment. The mechanisms that control or direct its responses are of great interest and may be particularly relevant to cancer. In cancer there appears to be a breakdown, quantitatively or qualitatively, in the response of cells to previously effective regulatory factors; this breakdown in response has not been readily explained by the appearance of unique structures or metabolic changes in the cancer cell. The extraordinarily complex cellular adjustments and their derangements may be referred, in part, to mechanisms described earlier; but other theories will have to be employed to explain how cells differentiate, respond to specific environmental changes, and maintain a precise relationship as to their location, number and function in various tissues and organs.

Although DNA exerts its influence on a metazoan cell through informational RNA and specific protein synthesis, external and situational factors play a large role in determining the fate of the cell. Cellular contacts, or local substances which diffuse into the cell, appear to modify and alter its growth and differentiation (196, 197). It is not known whether these or other factors influence DNA to direct the formation of mRNA, act directly on cytoplasmic structures, or whether both mechanisms could be directly involved. There is evidence that nuclear differentiation does occur during embryonic development. Nuclei have been obtained from frog embryos at varying stages of development, and transplanted into enucleated frog eggs. Normal embryonic development occurred with donor nuclei obtained prior to the blastula stage, but nuclei obtained from embryos in later stages develop into faulty and incomplete embryos (198, 199). It is suggested that these nuclei have undergone some change and are no longer totipotent. There is also morphological and biochemical evidence for the occurrence of chromosomal changes during differentiation. Swelling (puffs), or formation of "Balbiani rings," have been seen at specific loci of chromosomes at particular

periods of development (200, 201), and there is greater uptake of tritiated cytidine into RNA in the puffs than in other regions of the chromosome (202). Asynchrony of DNA replication has been described in different chromosomes and in different segments of single chromosomes of circulating human blood cells (203). A similar non-random and focalized pattern of DNA synthesis has been seen in chromosomes of Hela cells (204, 205). Consistent patterns in the behavior of the DNA of certain cells during replication may suggest that the DNA also has acquired altered functions that will result in changes in enzyme formation and specialization of cell function. This will result in the formation of certain products by cells, such as myosin by muscle cells, hemoglobin by erythrocytes, and steroids by cells of the adrenal cortex.

There is also evidence that cytoplasmic or environmental factors may play a role in cellular differentiation (206 to 210). These may operate through such mechanisms as adaptive enzyme formation or feed-back inhibition (211 to 215). "Small molecules" may exert a dual influence on enzyme synthesis either by inducing the formation of adaptive enzymes or by a repression of enzyme formation through their effects on informational RNA. *Enzyme induction* is the stimulation of synthesis of an enzyme; for example, β galactosidase is increased in *E. coli* in the presence of enzyme substrate, (lactose or other galactosides) (23, 216). *Enzyme repression* is a specific block in the formation of one or more of the enzymes in a specific biosynthetic pathway by an end product or metabolite formed in the pathway (217). Uracil, for example, inhibits the formation of enzymes required for the synthesis of its precursor, orotate (218). Repression
in a number of pathways of amino acid (219 to 221), purine and pyrimidine synthesis (218, 222).

Jacob & Monod (23, 38) have sugges
and induction of enzyme synthesis might function. The "structural genes" control the synthesis of specific mRNA which in turn controls synthesis of specific protein structures. "Regulatory" genes "control negatively the the transcription of certain specific structural genes without themselves contributing any structural information to the proteins." An "operon" consists of several "structural" genes which are controlled by a single "operator." According to this theory, "regulator genes" govern the synthesis of specific repressor substances, presumably RNA, which combine reversibly with "structural" genes of the DNA by virtue of possessing a complementary base sequence. The "repressor" blocks formation of specific mRNA by the structural gene, and as a result the synthesis of a specific protein is prevented. In an "inducible" enzyme system, presumably an excess of substrate requires increased enzyme formation. The blocking "repressor" is inactivated by specific combination with certain small molecules (the substrate or analogues thereof of the enzyme required), and is no longer able to block the synthesis of mRNA. The "repressor" must have high specificity for the "structural" genetic site on the DNA as well as for the enzyme which inactivates the "repressor" by coupling it with a small "inducer" molecule.

On the other hand, when the product (or its analogue) of the reaction sequence involving the enzyme accumulates, "repressible" enzyme systems are involved. The "repressor" itself is inactive, and is only capable of inhibiting RNA synthesis after it has been activated by combining specifically with the product of the reaction sequence (222).

Enzyme repression refers to the mechanism whereby enzyme synthesis is inhibited; by gradual dilution of the enzymes involved there is a slow decrease in the synthesis of the compounds involved in that metabolic sequence. Enzyme repression is to be distinguished from another mechanism of "negative feedback" control in which the end product of a biosynthetic reaction prevents the reaction from continuing formation by inhibiting in some manner an enzyme which is already present. In this sense an end product may be defined as the last molecule of a sequence (amino acid, nucleotide) before formation of macromolecules (protein, nucleic acid) (223). Remarkable efficiency is the rule in end product inhibition as it is always the first enzyme of a sequence which is inhibited, thus also controlling all the intermediates in that sequence (224, 225). Since the structure of the (end product) inhibitor and the substrate are invariably different, it is not surprising that the inhibitor may combine with the enzyme at a different site than that which is catalytically active for the substrate. These sites on the enzyme overlap in the case of "competitive inhibition," but do not when the end product noncompetitively inhibits the substrate's catalysis (225, 226). Indeed, the large size of most enzymes might presuppose that much of their make-up is concerned more with regulatory rather than purely catalytic function. Examples of end product inhibition are the "competitive inhibition" seen with cytidylic acid (227), by cytidylic pyrophosphate (226), by isoleucine, and by valine (228). Each of these end products decreases the activity of an enzyme necessary for its own synthesis. A dozen or more other examples of end product inhibition are known (225). This inhibition is a more sensitive, more rapid acting, and more easily reversible means of control than repression. It is primarily concerned with economy of small-molecule synthesis ("fine adjustment" control), while repression is concerned with overall economy of protein synthesis ("coarse adjustment" control) (223).

Alternative mechanisms for increasing enzyme activity following substrate administration have been proposed. Enzyme activity can be increased by activation of a pre-existing precursor of the enzyme by substrate; as examples, tyrosine in rats increases tyrosine-α-ketoglutarate transaminase activity (229), and tryptophane in rats increases trytophane pyrrolase activity (230). Another possible mechanism is that the substrate or product of the reaction may cause stabilization of an enzyme which continually is being synthesized and degraded. Thymidine or thymidylate may protect thymidylate kinase from being degraded, thereby leading to an accumulation of this enzyme (231). The level of thymidylate kinase activity in turn may have a regulatory role in DNA synthesis.

Cells presumably utilize processes, such as those described above, in the

course of differentiation, function, and response to hormonal and environmental factors. Certain cells, such as nervous and muscle cells, have already reached a high level of differentiation, and they are not able to divide or regenerate. Other cells, such as the bone marrow, and intestinal epithelium, multiply constantly. Some cells rarely divide, but are capable of regenerating to restore normal structures when needed. There are "germinal lines" or reserve cells in tissues capable of producing more differentiated cells when the proper stimulus occurs. Other tissues respond directly to specific humoral stimuli. This response is clearly shown in the growth and regression of certain organs due to the action of specific hormones.

Cell division is rarely found in the normal liver, but when a portion is removed, the remainder regenerates rapidly. The control of liver growth in the rat has been intensively studied. A substance, associated with albumin that is produced by the liver, apparently acts to inhibit cell division of liver parenchymal cells (232 to 234). When a portion of the liver is removed and the production of this fraction is decreased, cell division occurs until a sufficient concentration of the inhibitor is produced to prevent further growth. It has been suggested that mature granulocytes produce unstable inhibitors of mitosis in the granulocyte series acting in a manner analogous to the liver inhibiting factors (235, 236). Weiss has proposed a general concept that a tissue may control its own rate of growth by continuously producing rapidly degraded inhibitory substances "anti-templates" (237). Evidence is accumulating that erythropoietin's major mode of action is to cause undifferentiated stem cells to differentiate into pronormoblasts (238 to 241). There are admittedly local factors also involved in tissue growth; for example, the local tissue growth will stimulate balanced growth of blood vessels and stroma to support and nourish the increasing cell population.

The evidence for hormonal factors influencing "target" cell growth, function, and division are extensive. Often complex internal relationships exist; a decrease in the level of a specific peripheral hormone will cause the anterior pituitary to secrete the appropriate trophic hormone to restore the desired concentration of the peripheral hormone, for example, thyroid or adrenal cortical hormone. The natural plant hormones, the auxins and kinins, may greatly affect cell size and division (242 to 244). A relation has been shown between the presence of nucleic acid precursors and the activity of auxin (245). It is of interest that a mycoprotein plant extract, phytohemagglutinin, has recently been found to stimulate monocytes and large lymphocytes obtained from normal blood to divide when these cells are placed in short term culture (246). The mode of action of the phytohemagglutinin is not understood. Hormones control insect metamorphosis (247), and thyroxine is essential for tadpole metamorphosis, producing degeneration of certain structures and growth of others (248).

Sex hormones may cause a remarkable increase in mitotic activity in their several target organs, and have a lesser effect on other tissues, such as the skin (249).

The complex interplay of cells at all stages in the history of the organism

—in development and in regulation of cell growth, function, and degeneration—is only now being approached at an intracellular level. However, the results as they accumulate may be highly relevant to the chemical control of some forms of cancer. Certain cancers seem to show no response to any known regulatory mechanism, and these cancers grow in an unrestrained manner without evidence of differentiation or specific functional activity. Others however, retain susceptibility to environmental factors that are known to influence their tissue of origin, and altering the environment of these cancers may influence their growth and activity (250). Breast, endometrial, and prostatic cancers, as examples, may regress as a result of appropriate change in their normal environment. Adrenal steroids, which inhibit mesenchymal cells and destroy lymphocytes, can produce clinical benefit in patients with acute leukemia and chronic lymphatic leukemia. The discovery of specific factors that regulate other types of tissues may prove useful against cancers developing from these tissues.

Cellular resistance.—The development of resistance by normal cells to endogenous regulatory factors does not seem to occur in multicellular organisms, unless this change can be regarded as a mechanism for the development of cancer. Certain established neoplastic conditions, which may be initially susceptible to the action of a specific drug, ultimately become resistant and the disease follows its usual course despite continuing therapy (251). Resistance is apparently due to mutant cells which are able to propagate in the presence of the previously effective drug and cause recurrence of the disease. These mutations are generally stable and persist in the absence of the drug. Resistance to chemotherapy occurs in human as well as in animal cancers and leukemia, and is a major barrier to the control of these conditions. The mechanisms involved in the appearance of cellular resistance to specific drugs are of great importance in cancer chemotherapy (252).

EFFECTS OF DRUGS ON CELL GROWTH

The information recently available on cell growth and division provides a basis for determining the mechanism of action of anticancer drugs. However, interpretations of drug action at a cellular level are still tentative (253), and apparently conclusive results in one system may not be applicable to another (254). It is important to know how anticancer drugs act, but of perhaps greater value is the light they shed on cell biology (255 to 257). These findings, in turn, may prove relevant to an understanding of specific biochemical alterations in the cancer cell.

Polyfunctional alkylating agents.—Wheeler (258) has published a comprehensive analysis on the mechanism of action of the cytotoxic alkylating agents. Four types of compounds are of current interest: (*a*) mustards, including nitrogen mustard; (*b*) ethylenimines; (*c*) sulfonic esters; and, (*d*) diepoxides. In laboratory animals these drugs act on tissues that are being continually replaced, the bone marrow, the intestinal epithelium and germinal cells (259). Clinically these drugs are employed principally in the treatment of Hodgkin's disease, lymphosarcoma, chronic lymphocytic and chronic

myelocytic leukemia, and in carcinomas of the lung, ovary, and breast. While the various polyfunctional alkylating agents may produce temporary and partial clinical improvement in these diseases, the frequency of response varies with the different types of cancer and with the stages of the disease. The duration and degree of response are very difficult to quantitate satisfactorily. Evaluation of individual drugs is further complicated by variations in dosage and route of administration, and also the degree of toxicity induced in the patient. Thus, while there have been reports of enhanced therapeutic activity of chlorambucil in chronic lymphocytic leukemia (260), busulfan in chronic myelocytic leukemia, ethyl [*bis* (2,2-dimethyl-1-aziridinyl) phosphinyl] carbamate (AB 132) in carcinoma of the lung (261), L-phenylalanine mustard (Melphalan) in multiple myeloma (262), cyclophosphamide in acute leukemia in children (263), and thiotepa in breast cancer (264), the data provided to support the view that certain alkylating agents have selective effects against specific neoplastic conditions at doses relatively less injurious to normal tissues has not been conclusive. The available evidence still suggests that all the active polyfunctional alkylating agents have a similar cytotoxic mechanism of action and the same spectrum of therapeutic activity in the several types of neoplastic conditions responsive to these drugs (265). Schmidt (266) in his studies on cross resistance of animal tumors to alkylating agents also concludes: "These observations on cross-resistance at the experimental tumor level provide strong support for the concept advanced repeatedly by other investigators that many, if not all, alkylating agents have a basically similar mode of action."

The polyfunctional alkylating agents are highly reactive under appropriate conditions, and Wheeler (258) had defined the mechanism of alkylation as follows: "Alkylation is mediated through a cyclic sulfonium ion or a cyclic immonium ion which reacts at a negative or nucleophilic center. The chief nucleophilic centers which might be available for alkylation in biological systems are organic and inorganic anions, amino groups, and sulfide groups." The cytotoxic agents probably react indiscriminately with many cellular components, and sufficient concentrations are lethal to all types of cells. It is important, however, to discover the critical biochemical reactions which damage or destroy certain proliferating cells *in vivo* at sublethal doses to the host.

While mustard reacts with the free carboxyl, the mercapto, the amino and the imidazole groups of the proteins, and it is likely that some of the irregular deactivation of enzymes is due to alkylation, "this does not necessarily mean that the primary physiological effects of mustards are due to alkylation of proteins" (258). Random reactions with proteins or other cellular constituents may not be irreparable. In terms of lethal cellular injury, the important sites of mustard action appear to be on the nucleic acids. This is suggested by several lines of evidence. The alkylating agents are mutagenic (267, 268), carcinogenic (269), preferentially deactivate DNA-containing viruses (270) (although RNA viruses are also deactivated at higher concen-

trations), and inactivate the pneumococcal and H. influenzae transforming principles (271, 272). The effects of mustards on cells *in vivo*, as well as *in vitro*, have consisted of nuclear changes, with pycnosis or nuclear enlargement, fragmentation of chromosomes, and inhibition of mitosis. In some cases cytoplasmic growth may continue although cell division does not occur. The cytological changes seen in cells exposed to mustard vary with the cell type (273).

In *E. coli* sulfur mustard inhibited the synthesis of DNA, but with little effect on RNA synthesis (274). In a detailed review of the available evidence, Wheeler concludes (258) "data obtained both from *in vitro* and *in vivo* experiments show that mustards do inhibit the synthesis of nucleic acids with some variation dependent on the particular mustard and particular tissue. There is some evidence that DNA is inhibited more than RNA, and that there is little or no interference with the synthesis of acid-soluble nucleotides."

Trams *et al.* (275) have summarized suggested reactions for the polynucleotide-mustard alkylation reaction; they include: "(*a*) attack on the 3′,5′ linkage of DNA or RNA with disruption of the polymer; (*b*) alkylation of two adjacent phosphoryl hydroxyls within the polymer (intramolecular cross-linking); (*c*) alkylation of phosphoryl hydroxyls in adjacent chains (intra-molecular cross-linking); (*d*) alkylation of purine or pyrimidine bases with production and incorporation of 'base analogs' into the polymer; (*e*) alkylation of guanine at the 7-position with subsequent opening of the ring, loss of the base, and formation of an a-purinic acid-like structure; (*f*) formation of cyclic esters on the phosphate by dialkylation." Most recently, the major site of alkylation, when sulfur mustard or nitrogen mustard was incubated with RNA or DNA, was shown to be with the 7-nitrogen of guanine (276 to 279). Guanine was more actively alkylated than adenine or cytosine, and deoxyguanosine was more reactive than guanine or guanylic acid (280). The alkylation of the 7 position "increased the ease of scission of the bond between the guanine moiety and the sugar moiety . . . and mild alkali caused cleavage of the imidazole ring of the 7-alkyl guanine to yield the 4-amino-5-N-alkylformamido-pyrimidine derivative" (258). This selective reaction and the chemical alteration in guanine might lead to a break in the DNA molecule.

Another important reaction of the polyfunctional alkylating agents is cross-linking, with the two reactive groups of mustard joining together two points, either in the same or different DNA chains (281). This reaction is invoked to explain the greatly enhanced activity of the bifunctional as compared to the monofunctional mustard, although the latter is active at adequate doses. Cross-linkage by a polyfunctional alkylating agent may occur between bases in DNA; probably the principle cross-linking is between the guanine moieties. Steele (282) has suggested that the bifunctional alkylating agents may bind DNA to protein, by cross-linkage between the purines of DNA and carboxyls of glutamic and aspartic acids of proteins. By contrast the monofunctional compounds, which alkylate both DNA and protein

extensively, do not bind protein to DNA. Cross-linking appears to have a major role in the actions of mustards, but its exact significance remains unclear. It may interfere with the function of DNA, or prevent in some manner the separation of the twin strands of DNA and thus prevent normal replication. Thiotepa (283), methyl methane sulfonate, and epoxides also appear to react with guanine moieties.

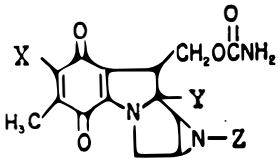
In whole cell and in *in vivo* studies, it has been shown that there is relatively little radioactively labeled alkylating agent recovered in DNA, and there is no definite correlation between the amount of alkylation of DNA and cytotoxicity (275, 284, 285). It has been suggested that DNA alteration may not be the primary injury responsible for cell damage, but a number of different mechanisms may be involved. Busch *et al.* (286) have suggested that "a primary action of the mustards is suppression of the biosynthesis of acid-soluble nuclear proteins because of the intranuclear binding of RNA by these alkylating agents." There are no data on the extent of alkylation of DNA in specific cell lines necessary to induce injury; but, for the present it seems reasonable to assume that DNA is far more vulnerable than other components of a cell. If random reactions with DNA result in certain alterations in its functions, the mechanism whereby this impairment causes cell injury and death requires analysis (287).

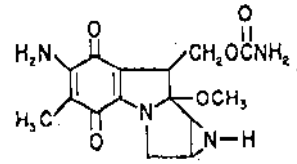
Non-lethal alterations produced by an alkylating agent result in mutation and carcinogenesis. Gelfant (111) has suggested that an alkylating agent does not inhibit DNA replication; in experiments with tritiated thymidine, DNA replication was not inhibited, but cell division after the G2 period was blocked. The random reactions of an alkylating agent with DNA may not block most of the sites of DNA replication, and the cytological abnormalities only become apparent later when the cell attempts to divide or is unable to enter mitosis. It seems possible also that the function that DNA performs in a specific cell will be related to the susceptibility of that cell to random injury to its DNA by an alkylating agent; thus certain proliferating cells, while presumably dividing at the same frequency as other types of cells, will be more susceptible. Certain types of neoplastic disease may fall in the latter category.

Mitomycin C

Mitomycin C, an antibiotic isolated from *Streptomyces caespitosus* by Wakaki *et al.* (288, 289), has been shown to produce specific damage to DNA (290). Its pharmacologic effects have been examined in rats, mice, dogs, and monkeys (291 to 293). It causes delayed deaths and weight loss, associated with hypoplasia of the bone marrow, lymphatic injury, and lesions of the intestinal epithelium. Mitomycin C disappears rapidly from the plasma, there has been no evidence of specific tissue localization, and one third or less of the injected dose is recovered in the urine. It is rapidly inactivated *in vitro* under anaerobic conditions, particularly by liver homogenates. Sokoloff *et al.* (294) has shown that growing rats may be partially protected from the weight inhibitory effect of Mitomycin C by cysteine and cysteamine.

Mitomycin C has a wide spectrum of antitumor activity against transplantable mouse and rat tumors (295), and has received extensive clinical trials against various forms of cancer in Japan and the United States (296 to 299). Therapeutic effects have been observed in lymphomas, chronic leukemias, and in some form of solid tumors, but the therapeutic role of Mitomycin C in clinical practices has not been established. The usual total dose, by the intravenous route in man is in the range of 1 mg/kg of body weight, given over a period of 4 to 10 or more daily injections. Mitomycin C may produce, at therapeutic doses in man, bone marrow depression with leukopenia, thrombocytopenia, bleeding and increased susceptibility to infection.





7-Amino-9a-methoxymitosane

I

(a) (b)

	MITOSANE Compound	MITOMYCIN C X	Y	Z
IA	Mitomycin A	H_3CO	OCH_3	H
IB	Mitomycin B	H_3CO	OH	CH_3
IC	Mitomycin C	H_2N	OCH_3	H
ID	Porfiromycin	N_2N	OCH_3	CH_3
IE	N-methyl-IA	H_3CO	OCH_3	CH_3

FIG. 3

The structure of Mitomycin C and related substances has been described in a series of papers from the American Cyanamid Company and Yale University. The compound mitiromycin (300), produced by *Streptomyces verticillatus*, was so named because it is found to be related to porfiromycin and mitomycin. The structure common to the mitomycins and porfiromycin has been given the trivial name mitosane, for the chemical formula 1, 1a, 2, 8, 8a, 8b-hexahydro-8-(hydroxy-methyl)-6, 8a-dimethoxy-5-methylazirino (2′, 3′-3, 4) pyrrolo (1, 2, -a) indole 4,7-dione carbamate (301 to 303). The structure of mitosane is shown in Figure 3a, and H in positions X, Y, and Z of mitosane are substituted in the several analogues listed below. Mitomycin C (Fig. 3b) is thus 7 amino, 9a methoxy mitosane. It is of interest that Mitomycin C has a quinone ring, a carbamate and an aziridine, each of which groups is known to have independent chemotherapeutic activity against some animal tumors.

Prior to the report on the structure of Mitomycin C, several studies on the mechanisms of action had been reported. Reich *et al.* (304), and Kersten & Rauen (305) utilizing several strains of bacteria, demonstrated that at

proper concentrations they continue to enlarge, but do not divide; if the bacteria are lysogenic, lysis occurs with release of virus; and if not, growth continues with formation of long filaments. The bacteria exposed to Mitomycin C failed to utilize tritiated thymidine, and were shown to lose DNA presumably by the breakdown of polymers of high molecular weight. The nuclear material was shown to undergo progressive fragmentation due to breakdown of DNA to smaller molecules. Mitomycin C did not affect polymerase *in vitro*. Viral RNA synthesis will proceed independently of DNA dependent cellular RNA synthesis, the latter being interrupted following the depolymerization of the cellular DNA (306).

In two mouse cell lines growing in tissue culture, L-929 fibroblast and S-91 melanoma, Mitomycin C caused the formation of giant cells, nuclear fragmentation with DNA-containing droplets in the cytoplasm, and partial depolymerization of nuclear DNA associated with a decrease in the intensity of nuclear Feulgen staining. Tritiated thymidine was still incorporated, to some extent, into the DNA of mitomycin treated cells; although the cellular DNA may be significantly injured, some DNA segments capable of replication might be left (307). It has been suggested that Mitomycin C produces effects similar to those of radiation (304).

The localization of mitomycin action in depolymerizing cellular DNA, the inhibition of replication of DNA in proliferating cells, the apparent radiomimetic action of mitomycin C, and the toxicological effects in animals, which are similar to those of the polyfunctional alkylating agents, all suggest that Mitomycin C may be acting as an alkylating agent. The fact that an aziridine group has been found in its structure would support this possibility. Mitomycin C, however, is considerably more active by weight in inhibiting cell growth than known monofunctional alkylating agents, and retains some biological activity after isolated hind-limb perfusion or after *in vitro* exposure to VX-2 carcinoma cells for one hour (308).

MERACTINOMYCIN (ACTINOMYCIN D)

Actinomycin, described by Waksman & Woodruff in 1940 (309), was the first crystalline antibiotic obtained from a *Streptomyces*; a number of representatives of the genus *Streptomyces* subsequently have also been found to produce actinomycins (310). Because of its high toxicity in animals, these substances were neglected until they were shown to inhibit the growth of transplantable tumor cells *in vivo* (311) and *in vitro* (312). This led to the clinical trial of a mixture of actinomycins (313). Subsequently, meractinomycin, a chemically definable substance of known structure, has been used in clinical work. Although a number of structurally related actinomycins have been examined in various laboratory systems and in patients with cancer, none have been shown to have advantages over meractinomycin.

In mice, rats, and dogs meractinomycin on systemic injection will produce damage to the bone marrow, lymphatic system and intestinal epithelium. This is associated with diarrhea, dehydration and bone marrow aplasia.

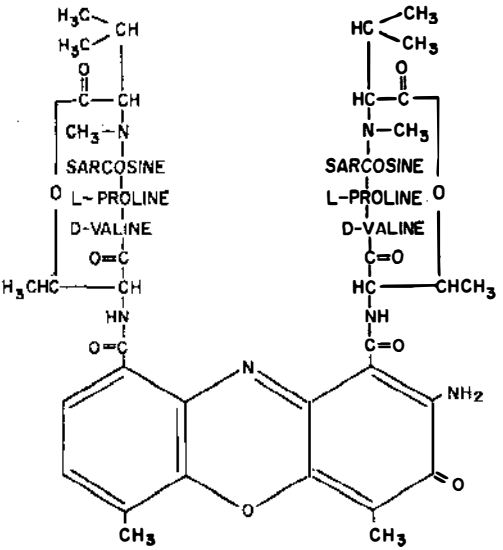
While these effects, involving tissues which are proliferating most rapidly, are similar to those produced by the alkylating agents and Mitomycin C, their evolution following meractinomycin appears to be more acute. The dose of meractinomycin in patients ranges from 0.5 to 2.0 mg/day, or a total dose of 0.05 to 0.075 mg/kg of body weight during a course of treatment. The toxic effects in man differ somewhat from those of the alkylating agents (314): there is definite redness and ulceration of the oral mucosa, occasional abdominal pain and diarrhea may occur, alopecia is more common, and in relation to the degree of damage to the digestive tract, bone marrow depression is less severe than that following the alkylating agents (315, 316). The actinomycins have been used principally in Wilms tumor in children (317), in the lymphomas, and in choriocarcinomas (318), and in combination with methotrexate and chlorambucil in testicular tumors (319). Meractinomycin may produce striking but temporary responses in Wilms tumors, and prolonged responses, possibly cures, in choriocarcinomas in females.

Brockmann (320) succeeded in separating several forms of actinomycin, and in elucidating the structure of actinomycin C_3. It consisted of a chromophoric portion, 2-amino-4, 6-dimethyl-3-oxo-phenoxazine-1, 9-dicarboxylic acid, which was named actinocin, bonded through its two carboxy groups to two peptide-lactone groups of equal structure. The actinocin portion of the molecule is constant, but several variations in the polypeptide chains have been produced. The structure of meractinomycin (or C_1) is shown in Figure 4. The sequence of amino acids alters the properties of actinomycin (321). The structural features of actinocin, and the 5 membered peptide chains and the lactone groups must be present for the full biological activity of actinomycin; substitution of the amino acids also modifies biological activity.

A correlation between the structure of meractinomycin and its proposed binding site with DNA has not been established. It was noted in 1958 that meractinomycin inhibited RNA, but not DNA synthesis in *B. subtilis* (322), and interfered with RNA synthesis in HeLa cells (323). Rounds *et al.* (324) observed that the loss of nuclear RNA in cultures of conjunctival and Hela cells treated with meractinomycin represented an effect similar to that of ribonuclease. Meractinomycin, however, was shown to be strongly bonded to DNA but not to RNA in solution (325), and in bacteria it inhibited the activity of an enzyme fraction capable of incorporating deoxynucleotides into DNA. It was suggested that meractinomycin might interfere with the action of DNA polymerase (326).

Using C^{14} labelled meractinomycin, Harbers *et al.* (327) found Ehrlich ascites cells took up the antibiotic rapidly; 80 per cent was found in the nucleus and 95 per cent of this was recovered from the DNA fraction. Meractinomycin inhibited the incorporation of tritiated cytidine into RNA, but did not interfere with tritiated thymidine incorporation into DNA of L cells in tissue culture (328). Reich *et al.* (328) further showed in strains L-929 and L-2 mouse fibroblasts that meractinomycin will completely uncouple the biosynthesis of normal cellular RNA from the biosynthesis of both DNA and

protein for prolonged periods and that the synthesis of RNA by these cells may be totally suppressed. The growth of the RNA containing Mengo virus is unaffected by meractinomycin at concentrations higher than those which inhibit RNA synthesis by host cells, whereas vaccinia, a DNA containing virus, is inhibited. The binding of meractinomycin to DNA is not irreversible since a DNA virus pre-incubated with meractinomycin may become viable when added to cells, presumably due to the competition of the cellular DNA for the viral bound meractinomycin (329). The ability to maintain protein synthesis after RNA production has been halted suggested that the ribosomal



Meractinomycin (ACTINOMYCIN D)

FIG. 4

informational template has considerable stability. Of further significance is that meractinomycin stopped all RNA production, indicating that cellular RNA formation is DNA dependent. In *in vitro* systems, using bacterial (330) and mammalian (331) enzymes capable of catalyzing the DNA-dependent synthesis of RNA, meractinomycin inhibited the synthesis of polyribonucleotides. "It seems probable that the enzymes catalyzing DNA biosynthesis and DNA-dependent RNA biosynthesis differ significantly in their respective stereochemical relationship to the DNA model (329)." A similar suggestion is made by Wheeler & Bennett (332) who suggest that meractinomycin, at physiological concentrations, binds to DNA and interfers with its template function in RNA production, although the synthesis of new DNA continues. It is

suggested that the imbalance resulting from DNA production and RNA inhibition leads to cell death. At high concentrations actinomycins interfere with *de novo* purine synthesis in *L. leichmanni*, and with the incorporation of a preformed purine (hypoxanthine) into RNA, but not DNA.

Kersten (333) showed that high concentrations of deoxyguanosine, and to a lesser extent guanine and adenine compounds, would alter the spectral properties of meractinomycin solutions. This has been confirmed (332), and DNA is also active in protecting against meractinomycin. Goldberg *et al.* (334) have found, in priming of DNA-dependent RNA synthesis, that the sensitivity to meractinomycin is a function of the G-C content of DNA primer; when DNA primers not containing deoxyguanosine residues are used, they do not bind meractinomycin (by spectral shift), nor is their priming of RNA synthesis inhibited.

In radioautographic studies using tritium labelled cytidine on Hela cells in tissue cultures, H^3 cytidine labelled the nuclear RNA, then the nycleolus, and later the cytoplasm. After meractinomycin a little tritiated cytidine went into DNA, presumably due to reduction to the deoxyriboside, but none was incorporated into RNA (335). Meractinomycin in tissue culture produces its earliest changes in the nucleolus, with a decrease in RNA. The nuclei become larger and the nucleoli are either absent or appear as small condensed masses. Budding and fragmentation of the nucleus are also seen. During the log-phase of Hela cell growth, many abnormal prophases accumulated; the chromosomes are swollen, shorter and thicker than those of the controls. The mitochondria remain normal in appearance (336). A strain of Hela cells was developed resistant to doses of meractinomycin which inhibit the sensitive strain.

Meractinomycin, thus, has been shown to form a complex with DNA involving selective binding at the G-C segments, with a specific block in DNA-dependent RNA synthesis. This block results in cell injury and death. Meractinomycin can be an important tool in elucidating the mechanism of RNA production and its role in the function of the cell.

5-Bromo-Iodo- and Chloro-2′-Deoxyuridine Analogues

The demonstration that certain 5-halogenated uracils inhibited bacterial growth (337), and that 5-iodo and 5-bromouracil were incorporated into DNA as thymine substitutes (338 to 342), led to important studies on the mechanism of action of the halogenated pyrimidines. Two important points should be noted about this group of drugs. First, whereas 5-iodo- and 5-bromo-uracil are incorporated into bacterial DNA, the halogenated bases and their ribosides are not incorporated by mammalian DNA (343, 344). The deoxyribosides derivatives (BUdR, IUdR), however, are utilized by mammalian cells (345), and are incorporated into their DNA (344, 346 to 350).

Secondly, the 5-bromo-, iodo- and chloro-2′-deoxyuridine derivatives have a categorically different mechanism of action from the 5-fluoro-2′-deoxyuridine, (FUdR). FUdR is not incorporated into DNA, but inhibits

de novo thymidine synthesis. The substitution of the heavier halogen atoms in the 5 position produces a functional thymine analogue. Szybalski (351) has noted that, whereas the van der Waals radii for hydrogen and fluorine are very close, being 1.2 and 1.35Å respectively, so that FU behaves more like uracil, the radius of the methyl group of thymine, 2.0Å, is more closely approximated by chlorine (1.8Å), bromine (1.95Å), and iodine (2.15Å) so that these latter 5-uracil derivatives act more like thymine. While certain quantitative differences have been noted between the effects of the 5-Br, 5-I, and 5-Cl analogues of 2′-deoxyuridine (351) they are qualitatively similar, and will be discussed as a group. Most of the work has been done with 5-iodo-2′-deoxyuridine (IUdR) (349).

Prusoff (346) synthesized IUdR in 1959, and demonstrated that it competitively antagonized the utilization of thymidine for the biosynthesis of the thymidylic acid portion of DNA, (347), and that it was also incorporated into the DNA of Ehrlich ascites cells (348). IUdR inhibited the growth of Sarcoma 180, and two strains of mouse leukemia *in vivo* (352), but it had no effect on the Walker rat tumor 256. Its antitumor effects were partially prevented by thymidine. It inhibited the growth of L5178 mouse leukemia *in vitro*, and about 35 per cent of DNA thymine was replaced by IU (353). I^{131} UdR was rapidly degraded *in vivo*, and, in mice, 71 per cent of the I^{131} appeared in the urine in 4 hours, and 91 per cent within 24 hours. About 83 per cent of the radioactivity appeared as free iodine (354). In dogs, IUdR in daily doses of 100 mg/kg intraperitoneally for 10 to 20 days caused anorexia, decrease in the formed elements of the blood, and desquamation of the intestinal epithelium (349). In patients, doses of IUdR ranging from 100 to 120 mg/kg given daily for a period of 5 to 6 days produced definite signs of toxicity, including nausea and vomiting, and effects on a variety of rapidly proliferating tissues. Bone marrow depression and moderate thrombocytopenia, oral lesions, and alopecia were found. When IUdR was infused systemically and thymidine given simultaneously into a small artery, protection in the area supplied by the artery was obtained (355, 356, 357). Intravenously, IUdR is rapidly destroyed in man, and the effects on tumor growth are modest, transient, and of no practical consequence. Toxic manifestations similar to IUdR were observed in two patients treated with continuous infusion of CUdR at a dose of approximately 20 to 40 mg/kg daily for 7 and 11 days (358).

Two mechanisms may be involved in the effect of IUdR on DNA metabolism. There is a decrease in the utilization of thymidine, thymidylic acid, or thymidine triphosphate into DNA (359), and in mouse Ehrlich ascites tumor and in four cases of human leukemia there appeared to be a block at the level of DNA-polymerase. IUdR is also phosphorylated and incorporated into DNA in place of thymidine, and its effects on cellular function may be more directly related to this mechanism. In support of this B- and I-UdR will protect cells and sand-dollar embryos against the thymidine deficiency produced by FUdR (360, 361); and, in the sand dollar embryo, develop-

mental disturbances develop later due to the presence of the abnormal pyrimidines (360). A combination of FUdR and BUdR has been used to increase the incorporation of BUdR in the DNA of *B. subtilis* (362), and IUdR has been given together with FUdR in an attempt to enhance antitumor activity, since the FUdR may increase the incorporation of IUdR into the tumor by blocking *de novo* thymidine synthesis (363). Since IUdR can interfere with the breakdown of FUdR *in vivo* (358, 364), the interpretation of these combination experiments *in vivo* is difficult.

BUdR is efficiently incorporated into DNA, and extensive substitutions of thymidine have been achieved. In a thymine-requiring mutant of *E. coli*, BU is incorporated but does not displace pre-existing T, and bacterial division is blocked (365). In tissue culture, up to 60 to 90 per cent replacement has been obtained during continuous exposure (366, 367). Hakala (367) obtained 93 to 100 per cent DNA thymine replacement with BUdR in human uterine fibroblasts and mouse fibroblasts when methotrexate was used to inhibit thymidine synthesis. Some of the cells continued to multiply but eventually succumbed. However, if the viable ones were returned to an antimetabolite free medium, some of the cells recovered.

BU apparently replaces T to give an adenine-bromouracil pair in DNA: BU does not appear to produce any major disturbance in base pairing, even when large amounts of T are replaced (368). BU is mutagenic in *E. coli* (369), and the biological effects are probably related to disturbances in the deoxyriboside pairing of adeninebromouracil (dA$\overline{\text{BU}}$) with resultant copying errors during replication of DNA (370).

There was no incorporation of guanine in the enzymatic replication of a DNA-like polymer containing only adenine and thymine residues (dAT). With an analogous polymer containing BU in place of T (dA$\overline{\text{BU}}$ primer), guanine was incorporated at a frequency ranging from 1 per 2,000 to 1 per 25,000 nucleotides polymerized. It is postulated that guanine is incorporated next to BU, and this may result from the bonding of guanine to a tautomeric form of BU resembling a 6-amino pyrimidine (371).

IUdR inhibited the growth of the DNA viruses herpes simplex and vaccinia in tissue culture (372). Kaufman (373, 374) has found that IUdR arrests established herpetic infections of the cornea in rabbits and man. This may be due to the incorporation of IUdR into the proliferating virus, or interference with its utilization of thymidine. FUdR did not have a therapeutic effect against herpes simplex, and iodouridine was without antiviral activity (375). Similar effects have been reported for herpes simplex in man following the application of an IUdR containing ointment (376).

Greer (377) first reported that BU incorporation into a thymine-deficient mutant of *E. coli* increased its sensitivity to ultraviolet irradiation. BUdR and IUdR also increased the radiation sensitivity of mammalian cells growing in tissue culture (378); IUdR was the most potent and CUdR the least effective radiosensitizer, although CUdR was most effective as a thymidine substitute in cells exposed to FUdR (379). Radio sensitization is dependent

on the incorporation of the halogenated pyrimidine into DNA (362); and 5-fluorouracil, which is not incorporated into DNA, does not sensitize to irradiation (380). Szybalski (351) suggests that the increased radiosensitivity of the halogenated DNA molecule is attributable to the postulated instability of the phosphate ester bonds between the BUdR moiety and the adjoining nucleotide, with resultant susceptibility to breakage. Radiation induced dehalogenation would also be augmented by the repulsion forces between the bromine and phosphate groups.

Under certain conditions some mammalian cells will grow indefinitely while continuously exposed to BUdR (351), whereas in other situations they fail to survive, as, for example, mouse leukemia lymphoblasts (L 5178) *in vitro* (381). Damaged cells may show polyploidy (382) and chromosomal breaks (383), and some cells may be enlarged (384). The disturbance may depend on the type of cell involved, and the functional role of the DNA in the particular cell. The halogenated pyrimidines presumably produce a defect in DNA, which can result in copying errors during replication; and because of the instability of the $A\overline{BU}$ pair, lead to chromosomal breaks. Mutations due to change in DNA can result in lethal disturbances in the cell (366).

Because of the rapid destruction of IUdR and BUdR *in vivo*, and poor incorporation into proliferating tissues and tumors (385), it has been suggested that the 5-iodo- and 5-bromo-2′ deoxycytidine derivatives may be more stable and thus more effective. In mice the halogenated deoxycytidine analogues are rapidly deaminated (386), but they have a more prolonged action in the rat (387, 388), with different patterns of tissue incorporation as compared to the deoxyuridine and analogues.

The drugs discussed thus far: alkylating agents, Mitomycin C, meractinomycin, and I-B-C-UDR- exert their principal effects on DNA. Each group appears to act somewhat differently, by alkylation, by depolymerization by an unknown mechanism, by bonding with DNA to block RNA synthesis specifically, and by the incorporation of abnormal bases into DNA. The points of attack can be visualized in Figure I.

5-Fluoropyrimidines

The fluorinated pyrimidines are not incorporated into DNA, but, depending on the analogue involved, are converted into nucleotides, incorporated into RNA, or inhibit thymidylate synthetase, an enzyme concerned with the methylation of deoxyuridylic acid to form thymidylic acid. The latter event results in a thymidine deficiency. Heidelberger *et al.* (389) first reported on the synthesis and biological activity of 5-fluorouracil. Subsequently a large number of fluorinated pyrimidine analogues were synthesized for trial as potential anticancer drugs (390 to 399). The most intensively studied of these have been 5-fluoro-orotic acid (FO), 5-fluorouracil (FU), 5-fluorouridine (FUR), and 5-fluoro-2′-deoxyuridine (FUdR). FU, FUR, and FUdR inhibited the growth of a number of transplantable tumors in animals, (391, 392, 400, 401) although not all tumors were inhibited (402 to 404).

Only FU and FUdR have had extensive trials against cancer in man. Clinical responses have been reported in a variety of cancers including those of the large bowel, breast, stomach, ovary, thyroid, pancreas, cervix, pharynx and urinary bladder (405 to 415). Even among the most responsive cancers (breast, large bowel, liver, stomach, cervix, ovary), there has been considerable variation in the reported response rates, ranging from 10 to 50 per cent. FUdR was reported to give a higher response rate in bowel and breast cancer (416), but this impression has not been confirmed (417). The duration of improvement produced by the fluorinated pyrimidines generally lasts only a few months; tumor regression usually is obtained at the expense of some degree of toxicity which may be severe, and there is no proof that tumor regression is associated with increased survival time. Toxicologic effects are observed in the more rapidly proliferating tissues; the bone marrow, the gastrointestinal tract, and sometimes the skin, conjunctivae and vaginal and other mucosal tissues. Severe toxicity is associated with extensive ulceration and hemorrhage of the gastrointestinal tract and aplasia of the marrow.

After daily injection of FU for seven days into mice with Ehrlich's ascites, the morphologic and histochemical changes that are seen in surviving tumor cells consist of cellular and nucleolar enlargement with doubling of RNA and protein content. There is clumping and irregular distribution of the DNA around nuclear vacuoles with a decrease in the amount of DNA per cell to less than half that in the controls. At the end of seven days, there is an 85 per cent inhibition of tumor growth and a comparable reduction in the mitotic index (418). FU has also been shown to inhibit growth of normal rat tissues including fetal growth (419), regeneration liver, growth hormone-induced growth of epiphyseal cartilage, and testosterone-stimulated growth of seminal vesicles (420). In patients receiving an adequate course of FU the marrow may become megaloblastic, along with associated changes in various epithelial cells and cancer cells (421). These morphologic changes are quite similar to those found in pernicious and other megaloblastic anemias resulting from nutritional deficiencies.

FU and especially FUdR have been found to be selectively localized in some susceptible experimental tumors; whereas, FO and FUR showed no such affinity for the tumors tested, and were sometimes found in higher concentration in various normal tissues (400, 422). No selective uptake of radioactivity was found in tumor tissue as compared to intestinal mucosa removed from patients several hours following administration of C^{14}-FUdR, although the nucleotide fraction from the tumor had a higher percentage of radioactivity (423), FU undergoes all the degradative (424, 425) and anabolic reactions (400, 422) of uracil with the important exception that presence of the fluorine atom prevents methylation of the 5 carbon of FUdRMP to form thymidylic acid (Figure 5). FU, as well as FUdR which is rapidly degraded to FU *in vivo* (425), is converted into FUR, the mono-, di-, and triphosphates of FUR (FURMP, FURDP, and FURTP); and into the monophosphate

(FUdRMP), but not the di- or triphosphates of FUdR (422). By these pathways, FU, but not FUdRMP, may be incorporated into RNA (400, 426, 427) but the fluorine in the 5 position prevents 5-methylation of FUdRMP, and FUdRMP cannot be incorporated into DNA. The pathways for cytosine and its derivatives are not included in Figure 5—partly for simplification and partly because 5-fluorocytosine (FC) is inactive in mammalian cells (398), and 5-fluoro-2′-deoxycytidine (FCdR) is rapidly deaminated to FUdR *in vivo* and by both normal and neoplastic tissues *in vitro* (358, 428). FCdRTP, however, will substitute for CdRTP and is apparently incorporated into DNA in an *in vitro* DNA-primed system with *E. coli* polymerase, and the possibility of producing DNA labelled with FC exists (398).

Although FCdR has occasionally shown greater activity in animals than FUdR, either in terms of antitumor effect or toxicity (398, 429 to 431), in initial studies in patients FCdR appears to act similarly to FUdR, at least when given by slow infusion (358, 432).

The fluorinated pyrimidines alter cell function by two main actions: affects on RNA, and DNA synthesis. FU and FUR can be incorporated into RNA as abnormal nucleotides, and also inhibit incorporation of orotic acid and uracil into RNA (422, 433, 434). While FU has no effect on total protein synthesis in *E. coli*, it may either block induced synthesis of specific enzymes (i.e., β-galactosidase) (427, 435), or result in other alterations in RNA and protein synthesis such as synthesis of abnormal enzymes (436 to 438), and

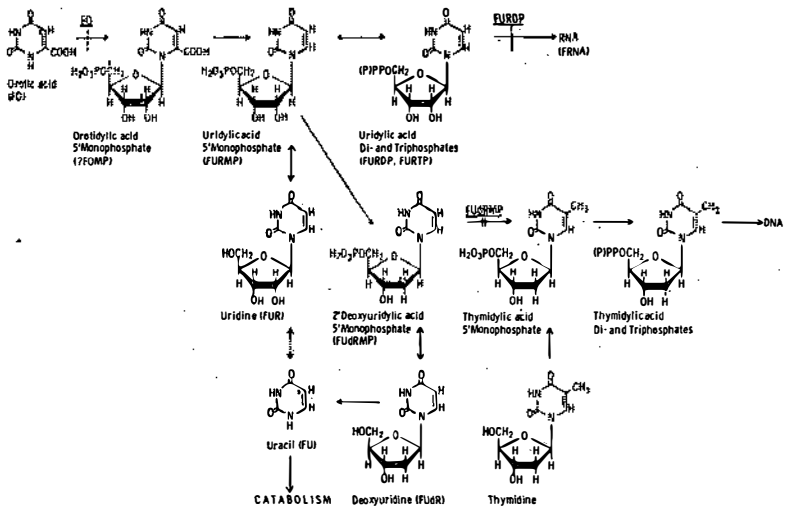


FIG. 5. Major anabolic pathways of the normal and the 5-fluorinated pyrimidine, and the latter's major sites of action. This is a composite scheme derived and modified from several sources (422, 517). The 5-fluorinated analogues which have either been synthesized or else isolated and identified are shown in parentheses. They differ from the normal compounds only in the replacement of the hydrogen by fluorine on the 5 carbon in the pyrimidine ring.

synthesis of a previously deficient enzyme (439). FU has also been shown to produce a temporary reversal of the defective phenotype of certain rII mutant T4 phages so that they will actively grow on strain K of *E. coli*, a strain on which they are ordinarily unable to develop (439). It was concluded that FU acts mainly by incorporation into T4 messenger-RNA in place of uracil at sites corresponding to AT in the phage DNA. In the m-RNA, FU acts partially like cytosine in coding for amino acids, thereby partially correcting the defective translation of genetic information from the mutant DNA of the rII phages.

Severe inhibition of protein synthesis has been produced in *E. coli*, whose ribosome content has been greatly reduced by magnesium starvation (440). Although addition of magnesium to the deprived cells in the absence of FU results in an exponential increase in ribosomes and an accelerating rate of protein synthesis, addition of FU with magnesium results in continued linear synthesis of protein. In other experiments with *E. coli* infected with T-even phages (DNA viruses), FU reduces phage production both by causing a selective inhibition of phage protein synthesis and by inhibiting formation of phage DNA; the latter effect is partially reversible with thymine (441). Aronson has suggested that in both phage-infected and magnesium starved cells, the ribosomes existing at the time of addition of FU may continue to function, but that the rate of new protein synthesis does not increase because FU prevents synthesis of new RNA necessary for new protein formation (440).

There is less evidence that the effect of the fluorinated pyrimidines on RNA and protein synthesis is important in mammalian cells, however, since continued protein formation has been demonstrated in several studies with concentrations of FU that inhibit DNA synthesis and cell proliferation (418, 422, 443, 444). FO and FUR are about 30 times as toxic by weight in man as FU or FUdR however, and this has not been satisfactorily explained (442). Although FO, like orotic acid, but unlike FU, is not extensively catabolized in mammalian systems (425), this difference in degradation has not been demonstrated for FUR. It remains possible that in man the toxicity of FO and FUR may in large part be due to alteration of RNA and protein metabolism.

The second and probably most important mechanism of action of the fluorinated pyrimidines is the inhibition of thymidylate synthetase by FUdRMP (Fig. 5). This enzyme, which catalyzes the methylation of 2′-deoxyuridylic acid (UdRMP) to form thymidylic acid, has been shown to be powerfully inhibited in enzyme systems obtained from *E. coli* (445), Ehrlich ascites cells (446), and in a mince prepared from an involved lymph node from a patient with Hodgkin's disease (423). The enzyme is not appreciably inhibited by FU, FUR, 5-fluorouridine-5′monophosphate (FURMP), FUdR or thymidylate (447). In the presence of ATP, however, FUdR did inhibit thymidylate synthetase because kinases contaminating the enzyme preparation employed produced FUdRMP (447).

Of the following fluorinated pyrimidines, FO, FU, FUR and FUdR, the

latter, which is the closest anabolite to FUdRMP, was most potent in preventing the incorporation of C^{14}-labelled formate (the specific precursor of the methyl group of thymine) into DNA thymine of intact Ehrlich ascites cells (433). FUdRMP itself is slightly less active than FUdR in intact cells because it presumably must be dephosphorylated prior to crossing the cell membrane (448). Uracil and FU gain entry into Ehrlich ascites cells by simple diffusion (449) while FUR and FUdR, like their normal counterparts, rapidly enter by passive transport (450).

The thymidine deficiency produced by thymidylate synthetase inhibition is presumably the main action of FUdR in producing cellular injury and death. It produces the syndrome of "thymineless death" due to specific inhibition of DNA synthesis in bacteria (445) and in mammalian cells (418, 444, 451, 452).

Treatment of *Vicia faba* root cells with FUdR resulted in the appearance of gaps in the chromosomes of cells reaching anaphase three to four hours later, presumably due to a failure of cells exposed to FUdR near the end of the phase of DNA synthesis to complete DNA replication (361). The G2 phase of the mitotic cycle between the end of DNA synthesis and division is about four to six hours in these cells. By six hours, only a few cells were reaching mitosis and many of these showed severe shattering of the chromosomes. These cells were in an earlier phase of DNA replication when synthesis of thymidylic acid was blocked by FUdR, and either halted their progress towards division entirely, or else barely reached mitosis with incomplete DNA. The latter cells then suffered severe chromosome damage during the coiling and tension processes of prophase and anaphase (361). Thymidine (or BUdR), given two or even three hours after FUdR, allows the cells to progress through mitosis with intact chromosomes visible at anaphase, presumably by allowing completion of the interrupted DNA synthesis.

The development of resistance to FU or FUdR has been noted in both experimental and human cancers that were initially responsive. In a cell-free enzyme system obtained from Ehrlich ascites cells, which had developed resistance to the fluorinated pyrimidines, the principal mechanism of resistance appeared to be an enzymic alteration in thymidylate synthetase, so that it could distinguish between the normal substrate UdRMP and the inhibitor FUdRMP (453 to 455). Although there was decreased conversion of FU into FURMP and incorporation into RNA in the resistant cells, this effect was not of sufficient magnitude to explain the resistance. The amount of FUdRMP produced was the same in both cell lines. In another FU-resistant Ehrlich ascites cell line, however, no change was found in the affinity of thymidylate synthetase for FUdRMP (456). Based on observations that in FU-resistant Ehrlich ascites cells there are decreased amounts of certain catalytic enzymes of uracil or FU including uridine kinase (456) and uridine phosphorylase (457), and in FU-resitsant *E. coli* there are decreased amounts of uridylic acid pyrophosphorylase (458), another mechanism of resistance has been proposed. This is a loss of capacity of resistant cells to carry out a "lethal synthesis" to form the nucleotide derivatives of FU which are the

active inhibitors (459). However, the primary significance of these observed decreases in enzyme activity is not clear; it has been suggested that they may be merely a contributory factor, and that the essential biochemical changes responsible for this type of resistance remain unknown (456, 460).

8-Azaguanine

In 1945 8-azaguanine (5-amino-7-hydroxy-1-v-triazolo [d]-pyrimidine) was synthesized and was shown to have antimicrobial activity (461). Kidder *et al.* demonstrated that it effectively inhibited the growth of certain mouse tumors, such as adenocarcinoma 755, whereas other tumors were unaffected (462, 463). A selective toxic action was shown also in the Brown-Pearce carcinoma in the rabbit. The tumor regressed at doses not toxic to the host (464). This drug has not received an adequate pharmacological investigation in laboratory animals, although Philips (465) noted that it caused a severe diuresis in dogs which led to dehydration, and which may have contributed to their death. There was no evidence of tissue damage in lethally intoxicated animals. In a preliminary trial in patients with cancer, 8-azaguanine was given intravenously at a dose of 200 mg/day. Total doses, ranging from 400 to to 1950 mg caused a toxic dermatitis, which appeared as a red macular rash over the major portions of the body, including the dorsum of the hands and feet. The rash often became confluent and in some cases progressed to the formation of vesicles and small bullae. Except for nausea and vomiting, no other signs of toxicity were noted. Therapeutic responses were not observed (466). Hall *et al.* (467) gave 8-azaguanine by local intra-arterial infusion into tumor-bearing areas. The dose varied from 0.75 to 3.0 mg/kg per day for 10 to 21 days. This treatment produced local and generalized skin rashes; transient tumor regression was noted in the infused area in 30 per cent of the patients.

The mechanism of action of 8-azaguanine has been summarized in several reviews (468, 469). The drug appears to follow the same metabolic pathways as guanine; 8-azaguanosine and 8-azaguanosine-5′-mono-, di-, and triphosphates have been found in soluble fractions from micro-organisms and from mouse tissues and neoplasms treated with 8-azaguanine (468 to 470). The drug can be incorporated in large amounts in place of guanine in *Bacillus cereus* (471, 472) and the sea-urchin embryo (473), and at much smaller levels in mammalian tissue and in tumors *in vivo* (468, 474, 475). While minute amounts have been recovered from the DNA of 8-azaguanine-treated *B. cereus*, it was not found in *E. coli* or T2 bacteriophage DNA (471).

A possible mechanism of 8-azaguanine action could be through conversion to 8-azaguanosine-5′-triphosphate, and as a guanosine triphosphate (GTP) analogue, it might interfere with the formation of peptide bonds in protein synthesis. However, 8-azaguanosine-5′-triphosphate, has been demonstrated to substitute for GTP (although it is less active) in the incorporation of amino acids into protein *in vivo* (476), and the effectiveness of the abnormal nucleotide as an adequate GTP substitute has also been demonstrated in several other systems (469).

The incorporation of 8-azaguanine into RNA may result in a defective informational RNA, and thus cause a disturbance in protein synthesis Creaser (477) observed that 8-azaguanine did not inhibit RNA synthesis in bacteria, but the formation of the enzymes β-galactosidase and catalase were inhibited. The inhibition could be reversed by guanine, xanthine and hypoxanthine; the 8-azaguanine in the pre-existing RNA was not replaced, but new, normal RNA was synthesized. Guanine has not protected against the 8-azaguanine effects in mammalian cells. In *B. cereus*, incorporation of amino acids into protoplasmic protein, but not cell-wall protein, was inhibited by 8-azaguanine (478). DNA synthesis was not inhibited immediately, but was blocked after the first doubling. There was also an increase in RNA formation (479), possibly explained by the "fraudulent" RNA giving rise to compensatory synthesis of more RNA (469).

RNA, containing 8-azaquanine, apparently interferes in the formation of enzymes. Together with the inhibition in amino acid incorporation into proteins (480), there was a decrease in lytic enzyme synthesis in *B. subtilis* (481), and inducible enzyme synthesis was also inhibited. In adrenalectomized rats, hydrocortisone increased the activities of hepatic glucose 6-phosphatase and fructose 1,6-diphosphatase and tryptophane-induced hepatic tryptophane pyrrolase; 8-azaguanine inhibited these rises in enzyme levels (482). In ad-3 mutants of *Neurospora*, which grow poorly in the absence of exogenous adenine, 8-azaguanine, as well as FU, caused a phenotypic, but not genotypic reversion. This is interpreted as a modification of the informational RNA by the abnormal analogue, thereby correcting the defective message, and resulting in restoration of normal enzyme activity (483). Protein synthesis is not affected nearly as much by 8-azaguanine in mouse leukemic cells *in vivo* as in bacteria, and Burdge & Mandel (484) suggest that the drug may inhibit mammalian cells by a more selective action on the formation of a specific but vital protein component.

Brockman (469) has summarized the studies on the mechanism of cellular resistance to 8-azaguanine and has concluded that this is frequently due to a decrease in inosine monophosphate-guanylic acid (IMP-GMP) pyrophosphorylase activity in the cell, so that 8-azaguanine cannot be utilized. This is confirmed in a recent report by Davidson *et al.* (485) in sensitive and resistant P 388 leukemia, and is supported by the observation that an ethyl derivative of 8-azaguanine (which cannot be converted to a nucleotide) has no biological activity in bacteria or animal tumors (486). High levels of guanine deaminase in tissues have been suggested as a basis for resistance to 8-azaguanine since the product of the reaction, 8-azaxanthine, has no antitumor activity (487). In a number of studies however, the level of guanine deaminase did not appear to be as important a factor in the occurrence of resistance (469).

The effects of 8-azaguanine on susceptible tumor cells are seen as a decrease in mitotic activity (464, 488), and cellular enlargement in the case of the Brown-Pearce carcinoma in the rabbit. In these *in vivo* studies, no toxic effects were observed on the jejunum, ileum or testis.

PUROMYCIN

Puromycin is an antibiotic produced by an actinomycete *Streptomyces alboniger.* Its chemical structure has been established as 6-dimethylamino-9-[3-deoxy-3-(*p*-methoxy-L-phenylalanylamino)-β-D-ribofuranosyl]-β-purine. (489 to 492) (Figure 6). Puromycin, thus, consists of dimethyladenine 3-amino-3-deoxy-D-ribose (known as the aminonucleoside of puromycin) and an amino acid *p*-methoxy-L-phenyl alanine. Puromycin and its aminonucleoside have shown considerable differences in their biological effects. Puromycin has irregular activity in experimental tumors (493), and its antitumor activity apparently resides in the aminonucleoside portion of the molecule (494, 495). Aminonucleoside was found to produce a progressive nephrotic

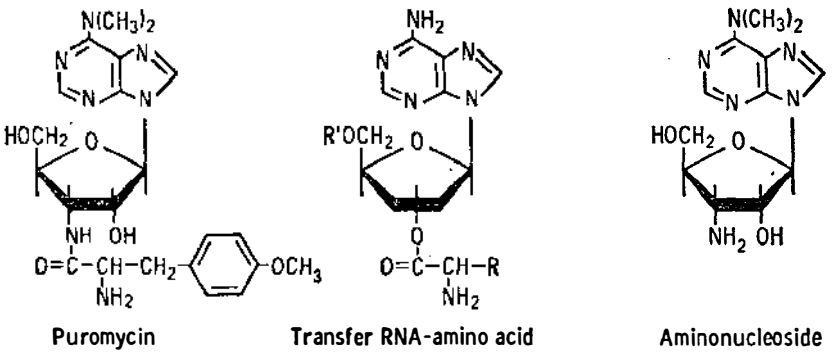


FIG. 6. Structures of puromycin, aminonucleoside and the amino acid-bearing end of transfer RNA (508, 500). R represents the remainder of the amino acid residue and R′ the remainder of the RNA polymer. The position of attachment of the amino acid on the ribosyl moiety of the terminal adenosine in the transfer-RNA is not yet known.

syndrome in the rat (496), and the renal lesion has been studied in great detail (497 to 501). The morphological disturbance apparently begins in the cells of the glomerulus, and proteinuria ensues. There is a subsequent loss of structure of the mitochondria in the proximal convoluted tubules (498). Puromycin is much less active than aminonucleoside in producing the renal lesion; and the specificity of the latter is shown by the findings that 3′ amino adenosine and 6-dimethyl adenosine do not produce renal toxicity (500). In monkeys, aminonucleoside, 10 to 15 mg/kg/day for seven days, produced a nephrotic syndrome, associated with extensive injury to the proximal convoluted tubules, and there was also evidence of liver damage (501). Aminonucleoside does not appear to produce its renal injury by an allergic mechanism, and it may act by inhibition of protein synthesis in the glomerular structure (502). Puromycin is effective against certain organisms such as *T. pyriformis* and *E. histolytica* (504), and mammalian cells *in vitro* (505) in

situations where its aminonucleoside is far less active. Puromycin toxicity against *T. pyriformis* can be reversed by guanylic acid (503).

Puromycin has been administered to patients with cancer. Doses of 250 to 750 mg/day to a maximum total course of 128 g caused nausea and vomiting, occasional diarrhea, but no consistent anticancer effects (506). Aminonucleoside, in a small series of patients, appeared to be more toxic. Daily doses of 3 to 4 mg/kg (100 to 250 mg) for 15 to 30 days caused severe proteinuria and desquamation of the ventral surfaces of the hands and feet. Proteinuria cleared when the drug was stopped (507). Again, there was no evidence of therapeutic activity. The explanation for the differences in the biological activities of puromycin and its aminonucleoside is not clear, but may be due to their metabolism *in vivo*, or to different effects on certain cellular systems. Most of the studies have been done with puromycin.

Puromycin was shown by Yarmolinsky & de la Haba (508) to inhibit the synthesis of protein in a cell-free preparation from rat liver. The block appears to be at the level of the transfer of the tRNA-activated amino acid complex to the ribosomal template preceding protein synthesis. Puromycin did not interfere with the rate of ATP generation, amino acid activation, nor the extent of tRNA binding with a labelled amino acid. It was not clear, however, whether puromycin inhibited the attachment of tRNA to the ribosome, or blocked the formation of the peptide chain. Puromycin stimulated the release of proteins from the ribosomes of ascites tumor cells (509), and reticulocytes (510). Puromycin, in an *E. coli* ribosome system, appears to promote the hydrolysis of the activated amino acid-tRNA complex, a reaction dependent on the presence of ribosomes, the transfer enzyme, GTP, phosphophenylpyruvate and pyruvic kinase. The poisoned ribosome, no longer able to transfer amino acids to protein, is still active in enzymatic deacylation. It is suggested that puromycin prevents the final condensation of the activated amino acids to peptide (59); tRNA is capable of being bound to the ribosome at levels of puromycin interfering with amino acid incorporation into protein (511). The structural resemblance between puromycin and the amino-acid bearing end of tRNAs has been noted (580) (Figure 6), but the precise mechanism of action of puromycin has not been established (512).

The block in protein synthesis is also produced by puromycin in more complex systems. A dose of 15 mg every hour times four intraperitoneally in adult rats caused a clear decrease in protein synthesis and, of the tissues examined, the thymus, kidney, and heart were most severely inhibited; RNA synthesis was not altered (513). Mueller *et al.* (514) showed that puromycin would inhibit the stimulation of uterine growth by estrogens in the castrated rat; this was related to the inhibition of protein synthesis, while phospholipid and RNA formation were not decreased. Protein synthesis thus appears to be an early and essential step in the action of estrogens on the uterus. In exploring the role of protein synthesis in cell division, Hela cells grown in tissue culture were synchronized by the addition of methotrexate and adenosine; when thymidine was added, DNA synthesis proceeded rapidly. Ten μg/ml of

puromycin in this system will promptly inhibit protein synthesis without irreversible injury to the cells; the culture may recover if puromycin is removed within three hours. In the synchronized cultures, puromycin added at various times in relation to the acceleration of DNA synthesis which follows the addition of thymidine, inhibited the acceleration phase. It is suggested that the competence of DNA to replicate is dependent on a process involving protein synthesis (505).

The preceding group of drugs, which include the fluorinated pyrimidines, 8-azaguanine, and puromycin, act on RNA by being incorporated into RNA or interfering with its function in protein synthesis. Drugs which produce this type of cellular disturbance, thus far, do not appear to have an important effect on cellular proliferation *in vivo*, nor a therapeutic action against cancer in man. Because of the conversion of FU to FUdR, its clinical activity appears to be related mainly to the inhibition of thymidylate synthetase.

Folic Acid Antagonists

There are a number of drugs that have been shown to interfere in various ways with the synthesis and interconversions of the purines and pyrimidines in nucleic acid metabolism. The induced deficiencies in the production of specific nucleotides and imbalance in the levels of various precursors cause disturbances in DNA replication, in RNA formation, and in the nucleotide containing coenzymes. The most important compounds active on these pathways are the folic acid antagonists, FUdR, 6 mercaptopurine and related compounds, azaserine and diazo-oxo-norleucine, 6 azauridine and azathymidine. It will not be feasible to describe all the effects of these drugs on biochemical pathways, nor on cell development, but they will be briefly noted; they have been described in detail elsewhere (515 to 517). The problems involved may be illustrated by a review of the folic acid antagonists which act in part to inhibit thymidine synthesis; FUDR, which was discussed earlier, also inhibits this synthetic process, but by a different mechanism.

The substitution of an amino group for the 4 hydroxy group of folic acid produces a highly active antimetabolite, 4 amino folic acid or aminopterin (518) (Figure 7). Compounds with other substitutions in the folic acid molecule have been studied; none has been found to be superior therapeutically to methotrexate (4 amino N 10 methyl folic acid; formerly called amethopterin) which is the anti folic compound used most widely in therapy (Figure 7). Methotrexate is about $\frac{1}{5}$ as active by weight as aminopterin.

In 1948, Farber *et al.* (519) reported the production of temporary remissions in acute leukemia in children with aminopterin, an observation which stimulated great efforts in antimetabolite chemotherapy of cancer. A series of analogues of aminopterin, as well as methotrexate, produced regression in a number of transplantable mouse tumors (520, 521). In laboratory animals, the 4 amino folic acid analogues caused delayed deaths with gastrointestinal lesions and bone marrow depression, indicating the toxic effects on rapidly proliferating tissues (522, 523).

The toxicological effects of methotrexate in man also result from damage to the normal rapidly proliferating tissues (i.e., bone marrow, epithelium of the digestive tract and the gonads). These effects may result in pancytopenia, megaloblastic anemia, bleeding, increased susceptibility to infection, diarrhea, ulceration of the digestive tract, alopecia and inhibition of spermatogenesis. Portal cirrhosis has been described in children on long-term therapy for acute leukemia (524, 525). Antifolic therapy during the early critical period of pregnancy may cause abortion or teratogenic effects in rats (526, 527) and humans (528 to 530) at doses not appreciably toxic to the mother.

Temporary remissions were induced with the folic acid antagonists in acute leukemia in 35 to 50 per cent of children, but only in 5 to 14 per cent of adults (531 to 533). Methotrexate has been shown to cause apparent *cures*, that is, no evidence of recurrent disease for six months to over five years after treatment thus far, in almost 50 per cent of women with metastatic choriocarcinoma (534 to 536). Striking regressions of localized tumors have sometimes occurred following methotrexate given by continuous infusion into the arterial supply to a tumor-bearing area (537). In this study, methotrexate was given intra-arterially in high dosage and its systemic effect partially counteracted by the intermittent intramuscular injection of citrovorum factor.

The mechanism of action of methotrexate in producing cellular injury must be considered in relation to the biological functions of folic acid. In order to exert its biological effect, folic acid (F) must be reduced to its active coenzyme form, 5, 6, 7, 8, tetrahydrofolic acid (FH_4) (Figure 7). This conversion occurs in two steps: the first is a TPNH-dependent reduction of F to 7, 8 dihydrofolic acid (FH_2), and the second a DPNH or TPNH-dependent reduction of FH_2 to FH_4 (517). Folic reductases are necessary for catalyzing both of these reductions (460, 517).

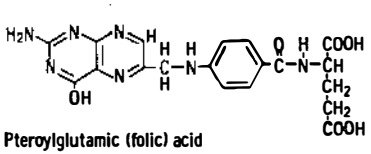
Several derivatives of FH_4 are involved in the transfer of 1-carbon groups in a number of essential reactions in the biosynthetic pathways of nucleic acids and amino acids (460, 517, 518, 538, 539). The 1-carbon moieties at various levels of oxidation (e.g. methanol, formaldehyde, or formic acid) are either formed *de novo* by neogenesis of methyl groups, or else may be derived from the metabolism of several amino acids such as methionine, glycine or serine (538). Derivatives of FH_4 transport the 1-carbon groups which may be in several forms including a formyl (–HC=O), a hydroxymethyl (–CH_2OH), or a formimino (–HC=NH) radical. The derivatives of FH_4 differ from each other in what form of 1-carbon unit they carry and whether it is attached to the N^5 or N^{10} or else may form a bridge between these two nitrogens (Figure 7).

Folinic acid is the N^5-formyl derivative (f^5FH_4); it is closely related, if not identical, to citrovorum factor (CF). The term leucovorin (Lederle CF) is retained for the synthetic form (537). In order for N^5-formyl FH_4 (f^5FH_4) to serve as a source of formyl groups for purine synthesis, it must be converted enzymatically in the presence of ATP into N^5, N^{10} anhydroformyltetra-

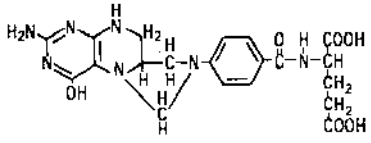
hydrofolic acid ($f^{5-10}FH_4$) (Figure 7). This, in turn, can be hydrated to another active N^{10} formyl derivative in the presence of cyclohydrolase (540).

The most important sites of action of the FH_4 derivatives are:

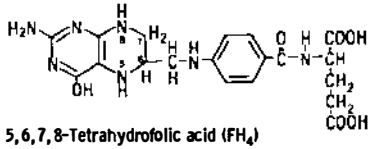
a) *de vovo* synthesis of purines—an N^5, N^{10}–anhydroformyl derivative ($f^{5-10}FH_4$) (Figure 7) is required for the transfer of formyl groups into the 8 and 2 position of the purine ring (e.g. the reactions respectively of glycineamide ribonucleotide (GAR)→formylglycineamide ribonucleotide (FGAR);



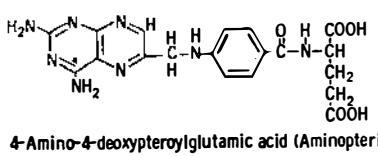
Pteroylglutamic (folic) acid



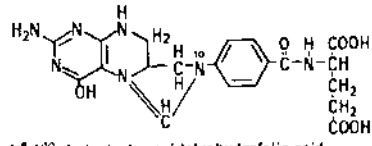
N^5, N^{10}-Methylene tetrahydrofolic acid ($h^{5-10}FH_4$)



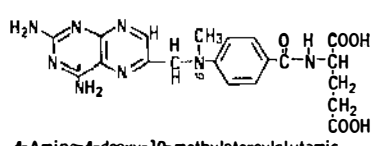
5,6,7,8-Tetrahydrofolic acid (FH_4)



4-Amino-4-deoxypteroylglutamic acid (Aminopterin)



N^5, N^{10}-Anhydroformyl tetrahydrofolic acid ($f^{5-10}FH_4$)



4-Amino-4-deoxy-10-methylpteroylglutamic acid (Amethopterin; methotrexate; MTX)

FIG. 7. Structures of folic acid, tetrahydrofolic acid and several of its active derivatives, and two folic acid antagonists, aminopterin and methotrexate.

and 5-amino-4-imidazole carboxamide ribonucleotide (AICAR)→formyl, 5-amino-4-imidazole carboxamide ribonucleotide (FAICAR).

b) Pyrimidine synthesis—an N^5, N^{10}-methylene derivative ($h^{5-10}FH_4$) (Figure 7) is required for the methylation of deoxyuridylic acid in the 5 position to form thymidylic acid. In this reaction, dihydrofolic acid (7, 8-FH_2) is formed by dehydrogenation of $h^{5-10}FH_4$; as a consequence, continued action of dihydrofolic acid reductase is required for regeneration of FH_4 (541 to 543).

c) Other 1-carbon transfers—although the action of FH_4 derivatives on purine and pyrimidine synthesis are of most significance in regard to discussion of drug action in proliferating cells, FH_4 derivatives also are involved in the transfer of 1-carbon groups in other important biological reactions including the synthesis of various amino acids, co-enzymes, and other mate-

rials (460, 517, 544), as well as the catabolism of purines (517) and L-histidine (545 to 548).

The principal action of methotrexate is to inhibit folic acid reductase, thereby preventing both steps in the formation of FH_4 directly from F as well as the regeneration of FH_4 from FH_2 that is formed as a byproduct in the conversion of deoxyuridylic to thymidylic acid. The affinity of folic acid reductase for these antimetabolites is far greater than its affinity for the normal substrates (F and FH_2); in one system using sheep liver, the affinity of the enzyme for methotrexate was almost one thousand times that for folic acid (549). Methotrexate does not bind irreversibly to folic acid reductase and inactivate it as was once proposed (550); rather, it is because of the remarkable affinity of the enzyme for methotrexate that even very large doses of folic acid given simultaneously, fail to reverse the toxic effects of methotrexate *in vivo*. If folic acid is given one hour prior to methotrexate, there is prevention of the toxic effects, since this allows time for the conversion of folic acid to active derivatives of FH_4 (551 to 556). CF, however, if given at the same time or shortly after methotrexate will prevent its toxic effects (537, 557), since CF (N^5-formyl FH_4) is a derivative of the product (FH_4) of the blocked reaction. CF, in turn, may be enzymatically converted *in vivo* into at least one other FH_4 derivative ($f^{5-10}FH_4$) (540). CF probably does not release folic acid reductase from its inhibition by methotrexate.

Studies with Sarcoma 180 cells and with mouse (557 to 559) and human (560, 561) leukemic cells have shown that there is a correlation between their folic acid reductase content and their sensitivity to inhibition by methotrexate (460). In these studies, sensitive cells had a low amount of folic acid reductase and utilized folic acid inefficiently in comparison with folinic acid, whereas cells resistant to methotrexate had a high folic acid reductase content (562, 563). Other studies with mouse neoplastic cells in culture, however, have failed to reveal any significant differences in the level of folic acid reductase activity between cells which were sensitive and those highly resistant to methotrexate (460). Furthermore, acquired resistance exhibited by some bacteria (564 to 566) and by mouse leukemic cells in culture (559), which are inhibited only by 100,000 times or greater the concentration of methotrexate necessary to inhibit the parent strain, cannot be explained by an increased folic acid reductase activity or by a more efficient conversion of folic acid to its functional forms. Thus, other as yet unexplained mechanisms of resistance to methotrexate must exist to allow the resistant cell to continue to form folic acid-derived co-enzymes which are clearly still essential for cell life (517).

Methotrexate is itself not reduced by folic acid reductase (549, 567), and there is no evidence that there is any formation of a biologically active derivative of methotrexate which might conceivably either substitute for, or compete with FH_4 or derivatives of the latter (517, 568).

Methotrexate is very resistant to degradation or any type of metabolic alteration *in vivo*, being largely excreted unchanged in the urine (569, 570).

Some methotrexate firmly bound to folic acid reductase is retained for long periods (up to eight months) in organs such as the liver and kidney with a low rate of cell division, whereas the drug is lost more rapidly from the intestinal mucosa, as a result of rapid cell renewal (570, 571). Methotrexate exhibits its major toxicity in rapidly proliferating cells; tissues with low mitotic activity, such as the liver parenchyma, are presumably unharmed by long-sustained inhibition of folic acid reductase activity, whereas the enzyme is essential for mitotically active cells (571).

There is good evidence that inhibition of DNA synthesis (largely a result of inhibition of endogenous thymidylic acid synthesis) is the most critical of the several effects of methotrexate in damaging proliferating cells (135, 460, 572 to 576). Grant showed that while methotexate inhibited DNA synthesis, there was no effect on the incorporation of precursors into RNA or the amount of RNA in *Rana pipens* embryos (572).

Thymidine-deficient growth of cultured Hela cells has also been clearly shown to be cause by methotrexate (135, 576). Rueckert and Mueller showed that Hela cells grown in a medium containing methotrexate and adenosine rapidly ceased to divide, but continued to increase in size for about seventy-two hours. RNA and protein synthesis continued at a normal rate for about sixteen hours (about two-thirds of the generation time), but thereafter the rate declined rapidly. RNA accumulation stopped entirely after thirty-six hours while protein synthesis continued at a very slow rate. Addition of thymidine up to about sixteen to twenty hours allowed the cells to recover and resume their normal proliferation rate; after longer periods, methotrexate damage was irreversible, even with added thymidine. The Hela cells could be made to divide synchronously by exposing them to methotrexate for about sixteen hours and then adding thymidine (135). Whereas in control cultures, only 30 per cent of logarithmically growing Hela cells are engaged in DNA synthesis (S phase) at any one time, growth in methotrexate for sixteen hours permitted the majority of cells to progress through the G1 phase so that they were also ready to initiate DNA synthesis upon replacement of the thymidine deficiency. The reversal of the thymidineless state resulted in initiation of DNA synthesis in 90 per cent of the cells, first at a slow rate and then at an accelerated rate after two hours. A wave of cell divisions followed after completion of DNA synthesis.

These experiments demonstrating that methotrexate arrests dividing cells in interphase due to inhibition of DNA synthesis clearly conflict with other results from which it has been concluded that folic acid antagonists act as metaphase inhibitors (129, 577 to 583). In this regard, Gelfant has emphasized that a wide variety of chemicals, including distilled water, cause metaphase inhibition in dividing chick cells grown in culture, and that the short-term conditions of the *in vitro* experiments in which methotrexate caused metaphase inhibition cast doubt on their significance (574). Further, several *in vivo* experiments have shown that folic acid antagonists inhibit cell division in interphase (584, 585). Under physiological condi-

tions, it may be concluded that methotrexate stops cell division in interphase due to inhibition of DNA synthesis. This effect is primarily due to a block in the synthesis of thymidylic acid, but may be in part due to inhibition in endogenous purine synthesis. In order to achieve a "thymidineless state" that could be reversed by adding thymidine, Rueckert & Mueller (575) supplemented their "methotrexate media" with adenosine.

Miscellaneous Antimetabolites Interfering with Purine and Pyrimidine Synthesis and Metabolism

Many other antimetabolites influencing purine and pyrimidine metabolism exist; they have been reviewed most recently by Brockman (469). The compounds in this group are numerous and their sites of action varied. The effects of several antimetabolites which are currently of interest as anticancer compounds will be briefly summarized.

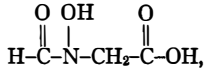
Azaserine and DON.—Azaserine (o-diazoacetyl-L-serine) and DON (6-diazo-5-oxo-L-norleucine) were isolated from broth filtrates of a streptomyces and their chemical structures were determined in 1954 (586), and 1956 (587) respectively. These drugs are naturally occurring diazo compounds closely related in structure in glutamine. Azaserine was initially detected in a broth filtrate because of its activity against transplantable mouse tumors. In subsequent clinical trials against various forms of cancer in man, both azaserine and DON showed weak to negligible therapeutic activity (588, 589). While their mechanisms of action on cells are in general very similar, and in most situations DON is about 20 to 40 times as active by weight as azaserine, greater differences in activities have been noted in specific situations (469).

The major action of azaserine and DON is to block the conversion of formylglycineamide ribotide (FGAR) to the corresponding amidine (FGAM), a step involving glutamine and a phosphoribosyl-formyl-glycineamidine synthetase. The antimetabolites are tightly bound to this enzyme, so that in an *in vitro* system, if they react with the phosphoribosyl-formyl-glycineamidine synthetase prior to the addition of glutamine, they cannot be displaced (590). The glutamine antagonists also block the reaction of 5 phosphoribosyl-1-pyrophosphate with glutamine to form 5-phosphoribosylamine, a purine precursor, but this system is not as sensitive to the antimetabolites as the preceding one (591). Other systems in which the glutamine antagonists inhibit transamination reactions involving the transfer of the amide nitrogen of glutamine include the conversion of uridylic to cytidylic acid, and of xanthylic to guanylic acid (592).

Ehrlich ascites cells, exposed for six days *in vivo* to azaserine develop enlarged nuclei, with a decrease in DNA content, and cell division is inhibited (593). Azaserine and DON, by interfering with amination reactions, block *de novo* purine synthesis in susceptible cells, and produce a deficiency in essential substances for cell growth and function.

Hadacidin.—This new substance, isolated from broth filtrates of *Penicil-*

lium frequentans, was shown to inhibit Toolan's human adenocarcinoma (HAD 1) growing on the chorioallantoic membrane of the chick embryo (594, 595). The active substance was found to be N-formyl-N-hydroxyglycine,



and it was given the trivial name hadacidin (596). It has no important antitumor activity in tumor-bearing mice and rats, and large doses, in excess of 2 g/kg/day, are tolerated by mice and rats (597). It is teratogenic in the chick embryo, and produces severe fetal abnormalities when administered to the pregnant rat in the lethal dose range (598). In preliminary clinical trials, doses of 5 to 10 g/day and higher for two to three weeks produced a decrease in the elevated leucocyte count in acute and chronic granulocytic leukemia; the clinical response was not entirely satisfactory, and the dose caused some nausea and diarrhea (599).

Hadacidin has been shown in Ehrlich ascites tumor cells and in liver brei to compete with the normal substrate, L-aspartic acid, for the enzyme, adenylosuccinate synthetase resulting in the competitive inhibition of adenylosuccinate formation (600, 601). This is an essential step in the conversion of inosinate to adenylic acid, and adenylic acid formation is blocked by hadacidin. This inhibition is competitively reversed by L-aspartate. At higher concentrations, hadacidin also inhibited the formation of uridylic acid, presumably at a step prior to the formation of orotic acid, and related to the synthesis of carbamyl aspartate (602). Hadacidin did not inhibit the incorporation of amino acids into proteins.

Mercaptopurine.—Of a large series of purine analogues studied, mercaptopurine (6-mercaptopurine; 6 MP) synthesized in 1952 (603), was highly effective against transplantable mouse tumors (604). In clinical trials, it produced temporary remissions in acute leukemia in children and adults, and suppressed the manifestations of chronic myelocytic leukemia for long periods (605, 606). It continues to be an effective and widely used drug in these conditions.

Mercaptopurine is readily converted by susceptible cells to 6 MP nucleotide, and in this form it has been shown to interfere with various mechanisms concerned with purine metabolism. None of these mechanisms, however, are necessarily solely responsible for the inhibition of cell growth caused by mercaptopurine. Its major effect appears to be related to a block in the interconversion of purines from inosinate at the nucleotide level (607).

Mercaptopurine blocks the step from inosinate to adenylosuccinate by acting on the enzyme adenylosuccinate synthetase, and thus produces an adenylic acid deficiency. Hadacidin acts at this point also, competing with the substrate of the enzyme, L-aspartate. Also mercaptopurine interferes with the step from inosinate to xanthylate by inhibiting inosinate dehydrogenase, and thus guanylic acid formation is inhibited (607). This disturb-

ance in the interconversion of the purines interferes with cell growth. Mercaptopurine also inhibited DPN synthesis (608), and may, by means of a feed-back mechanism inhibit the formation of a purine precursor, phosphoribosylamine (609). There is no evidence that mercaptopurine is incorporated to any extensive degree into RNA or DNA. In contrast, thioguanine, which appears to be similar to mercaptopurine in its antitumor activity in animals and man, is reported to be incorporated in RNA and DNA. It is postulated that thioguanine may act by producing an abnormal DNA, and in resistant cells, less thioguanine is incorporated into DNA (610).

Resistance to mercaptopurine develops readily in laboratory animals, and in acute leukemia in man. As is the case with 8-azaguanine a major mechanism in resistant cells is a decrease in their ability to convert mercaptopurine to its nucleotide, due to decrease in IMP pyrophosphorylase activity.

6-azauridine.—Handschumacher *et al.* (515) have recently reviewed the current status of 6-azauridine (6AzUR). 6-azauracil (6AzU) is not readily converted to a nucleotide by mammalian cells, and it produced serious central nervous system disturbances during a clinical trial. 6AzUR also inhibited the growth of animal tumors, but it is less toxic than 6AzU. 6AzUR is well tolerated by man and intravenous doses of 120 to 240 mg/kg, and as high as 600 mg/kg daily have been administered in clinical trials (611). While 6AzUR produced bone marrow depression in dogs, it appears to have a negligible effect on the human marrow at these doses. Incomplete responses were observed in patients with leukemia. Resistance developed, and this was correlated with the restored ability of the leukemic cell to decarboxylate orotic acid. It was shown that 6AzUR could be converted to 6-azauridylic acid, and thus act as a competitive inhibitor *in vitro* and *in vivo* of the enzyme orotic acid decarboxylase, blocking the conversion of orotydylic to uridylic acid. This results in an increased production and excretion of orotic acid and orotidine (611).

It is noted that in this group of antimetabolites are agents which specifically block pathways for purine and pyrimidine metabolism. FUDR blocks thymidylate synthetase to produce a thymidine deficiency; methotrexate interferes with the transfer of formyl groups to the 2 and 8 positions of the purine ring, and for the methylation of deoxyuridylic acid to form thymidylic acid; azaserine and DON block transamination reactions involving glutamine as a nitrogen donor, and thus inhibit purine synthesis; hadacidin interferes with the conversion of inosinate to adenylic acid; and 6-mercaptopurine blocks the pathways from inosinate to both adenylic and guanylic acids; and, finally, 6-azauridine prevents uridylic acid formation by inhibiting orotidylate synthetase. By these mechanisms, the cell is rendered deficient in essential materials necessary for growth.

MITOTIC INHIBITORS

An entirely different group of drugs are characterized by their action in blocking cell division in metaphase, without interfering with DNA replica-

tion or pre-mitotic events. The most important drugs in this group are alkaloids derived from plants; colchicine from the autumn crocus (*Colchicum autumnale*), podophyllotoxin from the powdered roots of the mandrake) (*Podophyllum peltatum*), and vincristine and vinblastine from the myrtle (*Vinca rosea*). The structures of these drugs are shown in Figure 8; the structure of the vinca alkaloids have been elucidated in the past year (612).

Colchicine and podophyllotoxin.—Colchicine has been reviewed in detail in a monograph by Eigsti & Dustin, published in 1955 (613). The literature

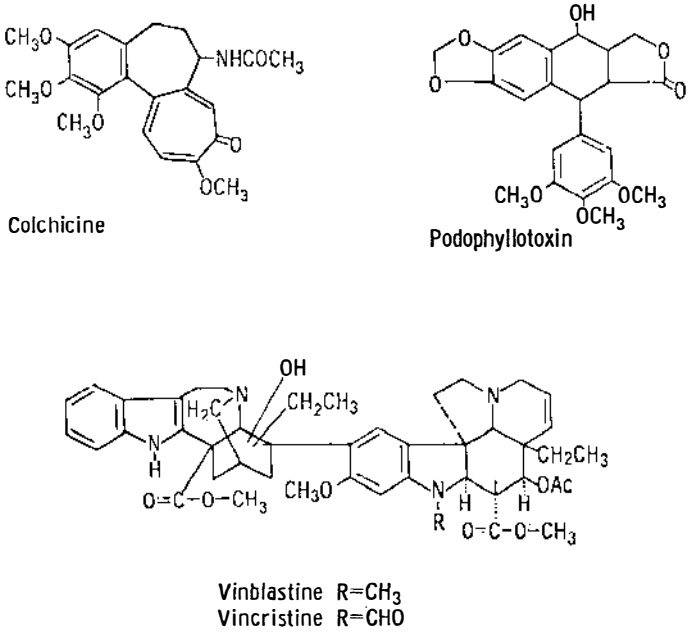


FIG. 8. Structures of several mitotic inhibitors: colchicine, podophyllotoxin, vinblastine and vincristine.

is extensive, but the exact site of action of colchicine has not been defined. Its most conspicuous effect is a production of metaphase arrest presumed to be due to "the change of the mitotic spindle from a polarized fibrous structure into an amorphous hyaline globule or a 'lake-like' body that is no longer capable of supporting the movement of the chromosome. Just how colchicine and other C-mitotic agents cause this damage is not clear, but each new category of metaphase poison tends to spawn a fresh theory (255)." In the most recent study of the colchicine effect, Sauaia & Mazia (614) used sea-urchin eggs and a colchicine analogue, demecolcine (desacetylmethylcolchicine). They observed that the asters were most sensitive, and that the spindle is subsequently disorganized. Demecolcine had no effect on

the ATPase in the spindle, and the isolated mitotic apparatus was not sensitive to demecolcine. This suggests that the action of colchicine is indirect, mediated by processes going on in the intact cell. The so-called C-mitosis, or colchicine mitosis may vary considerably from chromosomes lined up in the metaphase but unable to separate to the highly contracted chromosomes of the "ball-metaphases." The action of colchicine may actually involve the entire cell, altering the cytoplasmic viscosity. Padawer (615) has described specific changes in the mast cells of the rat produced by active analogues of colchicine and podophyllotoxin. Mast cells do not divide, but when exposed to these drugs they go from a round to a polymorphic form, and the nucleus is peripherally displaced. It is suggested that "colchicine directly or indirectly effects some contractile or expansile proteins perhaps present in most cells." The effect on cytoplasmic viscosity has also been studied by Nishimura & Baum (616, 617).

There is a close structure-function relationship in the action of colchicine on animal tumors (618). In laboratory animals colchicine produces delayed deaths with severe gastrointestinal disturbances, bone marrow depression (619), and weakness and atrophy of skeletal muscles (620). Colchicine is the classical treatment for gout, and it was recently suggested that it acts by reducing the inflammatory reaction, phagocytic activity of leucocytes, and lactic acid production in involved joints. (621). Colchicine has some activity in chronic myelocytic leukemia and Hodgkin's disease, but demecolcine has been more widely used because it appears to have less gastrointestinal toxicity. This drug, however, at toxic doses produces bone marrow depression, ulceration of the digestive tract, alopecia, and dermatitis (622).

Podophyllotoxin has not been as intensively studied, but its effects on cells appear to be similar to these of colchicine. It inhibits tumor growth (623), and there is a close structure-function relationship (624). The clinical activity of podophyllotoxin by systemic administration has not been adequately explored in man.

Vinblastine and vincristine.—The brief discussion of the other antimitotic alkaloids provides a background to the current interest in the vinca alkaloids. The two drugs of clinical interest are shown in Figure 8. The first alkaloid described, vinblastine, showed a wide spectrum of antitumor activity in laboratory animals (625, 626). Clinical trials were begun in 1960, and the drug has shown therapeutic activity in Hodgkin's disease, in some cases of acute leukemia and in choriocarcinoma in females (627 to 632). The usual dose is 0.10–0.15 mg/kg intravenously at weekly intervals. This dose is cumulative and may produce leucopenia and thrombocytopenia, with evidence of metaphase arrest in the bone marrow, and other toxic manifestations including nausea, diarrhea, paresthesias, loss of deep tendon reflexes, alopecia, and mental depression.

Vincristine is more active by weight, and the weekly intravenous dose ranges from 0.03 to 0.075 mg/kg. There are definite differences in the clinical effects of the two agents. Vincristine, in contrast to vinblastine, will

induce remissions in acute leukemia in children (633). It also causes less severe injury to the normal bone marrow in relation to its neurotoxic and gastrointestinal effects. The neurotoxicity consists of paresthesias, loss of deep tendon reflexes, palsies, peripheral neuritis, and severe constipation with ileus. Because of the lesser neurotoxicity, vinblastine appears to be more useful in Hodgkin's disease.

Vinblastine produces nuclear changes in a variety of cells, which are similar to those seen with colchicine (634 to 640). A range of nuclear effects were observed *in vivo* and *in vitro* with metaphase arrest, consisting of condensed nuclei, multi-nucleated cells, and nuclear cleavage and blebbing. With vincristine, Cardinali *et al.* (641) observed similar nuclear changes.

Since the vinca alkaloids fall into the group of antimitotic agents, differences in the therapeutic activity and toxicological effects of vinblastine and vincristine are difficult to explain. It has been suggested that these drugs act by a mechanism not related to their antimitotic effects. Vinblastine, in tissue culture, appeared to interfere with metabolic pathways leading from glutamic acid to urea, and with the citric acid cycle (626). Tryptophane and glutamic acid given, prior to vinblastine, to mice with Ehrlich ascites reduced the number of mitotic figures, suggesting some protective activity (638). Vaitkevicius *et al.* (639) noted some amelioration of vinblastine toxicity in patients when it was combined with glutamine. Protection was only partial, and the tolerated dose of vinblastine was increased about 30 per cent. The protective action of glutamine, however, could not be confirmed by Cardinali *et al.* (641).

Despite the differences in structure colchicine, podophyllotoxin, and vinca alkaloids have similar effects on cell division. A great deal more work is needed in order to determine if these drugs act on the dividing cell through a common mechanism, and if there is a clear dissociation between their antimitotic activity and their pharmacologic and antitumor activity.

STEROID HORMONES

Whereas the drugs discussed previously act on rapidly proliferating cells, the steroid hormones are individually more selective in their effects. Each hormone acts on specific tissues, and depending on the tissue involved, stimulates or inhibits its growth. The importance of the steroid hormones in cancer chemotherapy lies in the fact that cancers arising from tissues modified by the steroid hormones may retain some of the hormonal responsiveness of their tissue of origin. Thus, alterations in the hormonal environment may increase the growth rate or cause the cancer to regress, depending on the cancer's primary site and the properties it retains from its tissue of origin.

The steroid hormones are in a complex relationship with each other, with hormones produced by other target organs, and with hormones secreted by the anterior pituitary gland. Although steroids that depress tumor growth may act in part by inhibiting anterior pituitary secretion of trophic hormones (i.e. gonadotrophin, prolactin, ACTH, growth hormone) (642 to

644), this review is only concerned with their action on peripheral target tissues. It will not be possible to discuss the ablative forms of hormonal therapy which have proven useful in treating certain cancers (645 to 651), nor the extensive work on the metabolic transformation of steroids *in vivo* in both normal subjects and in those with cancer (652 to 665), or *in vitro* in normal and malignant cells (666 to 673). Only the hormones of greatest clinical value in the treatment of cancer will be discussed: androgens, estrogens, progestins, and corticosteroids (Fig. 9). When used appropriately, these steroids have produced responses in breast, prostatic, and endometrial cancers, and in acute leukemia and chronic lymphatic leukemia. Discussion will be limited to possible mechanisms of action relating to either inhibition or stimulation of tumor growth. Several reviews have been published that deal more comprehensively with the diverse biological activities of steroid hormones (654, 674 to 677).

Androgens will cause objective temporary regression in about 20 per cent of patients with breast cancer (678); but sometimes, they accelerate the growth of breast (678) and prostatic cancer (677). Depending in part on the age of the patient, androgens stimulate the growth of certain normal structures (accessory male sex tissues, body hair-follicles, sebaceous glands, larynx, erythropoietic tissue and long bone epiphyses), while other tissues fail to develop normally or become atrophic (breast, uterus, vaginal mucosa) (674, 676, 677).

Estrogens are effective in the treatment of carcinoma of the prostate (647, 648, 679, 680, 681), and in metastatic mammary cancer in post-menopausal patients (678). They may also accelerate the growth of mammary cancer, especially in patients who are pre-menopausal or less than five years post-menopausal. Estrogenic effects on normal tissues in females include development of the nipples, breast, genitalia and pubic hair in adolescence; while in adults, proliferation of endometrium, myometrium and the vaginal mucosa occurs. In males, feminization effects may occur involving the breast, skin, hair, fat distribution, and decrease in size of genitalia. (676, 677, 680).

Steroid therapy has produced severe degenerative changes in prostatic cancer cells sensitive to estrogens (682 to 685), and in breast cancer cells sensitive to either estrogens (686 to 690) or androgens (688). These include cytoplasmic vacuole formation, nuclear hyperchromaticity and pyknosis, and rupture or dissolution of the cells. Considerable variation has been noted among tumors of different patients and even within the same tumor, with some cells showing marked changes while others are apparently unaffected.

The stimulation of growth induced by the sex steroids of their normal target tissues is associated with characteristic cytological changes. Androgenic stimulation of castrated rats causes increase in size of the nuclei with restoration of the normal chromatin pattern as well as considerable cytoplasmic growth in epithelial cells of the prostate and seminal vesicles. There

is enlargement of the Golgi apparatus, appearance of abundant mitochondria, granules and vesicles, and the glandular lumina become filled with secretions (686, 687). Estrogen administration causes considerable nuclear and cytoplasmic growth of rat endometrial cells associated with increased amounts on RNA, glycogen, and glycoprotein (688, 689). The sex hormones also greatly stimulate mitosis in their target organs (162, 688), and may have a lesser effect on the distant tissues such as the epidermis.

In the target organs showing a marked increase in mitotic activity, there is a latent period of several days during which cellular hypertrophy precedes

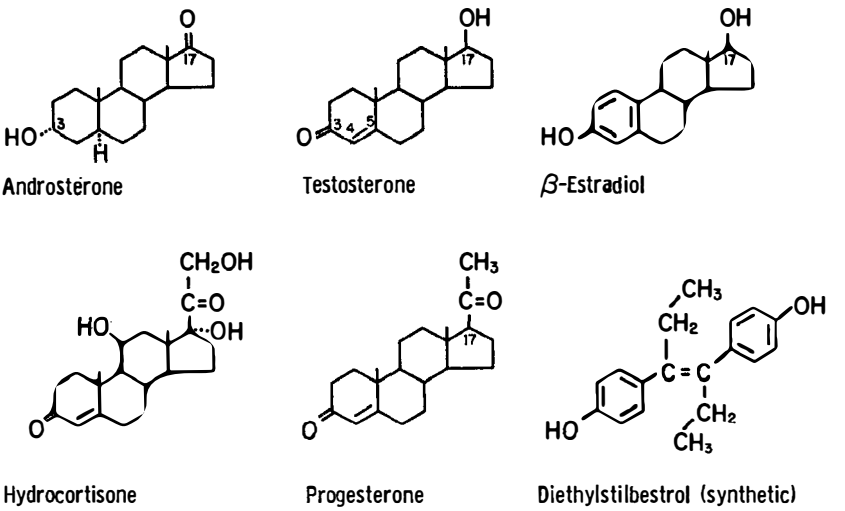


FIG. 9. Structures of testosterone; one of its metabolic conversion products androsterone; β estradiol, the normally secreted ovarian hormone; diethylstilbestrol, a synthetic non-steroidal compound which has potent estrogenic activity; progesterone; and hydrocortisone (17α-hydroxycorticosterone or cortisol).

mitosis (162). The target organs in which this sequence occurs normally have low mitotic indices, and the latent period is presumably necessary for synthesis of proteins and enzymes required for cell division. The non-target tissues (such as the epidermis) show only a several-fold increase of mitoses which appears within a few hours; these tissues normally have a fairly high mitotic index.

Progestational agents in large dosage cause temporary objective improvement in about one fourth of patients with inoperable carcinoma of the endometrium (691 to 693). Progesterone is necessary for the maturation and secretory activity of normal endometrium, and in addition produces characteristic changes in the vaginal epithelium (694, 695).

Adrenal corticosteroids will sometimes cause striking regression of dis-

ease in patients with lymphomas or leukemias (696 to 701). Acute lymphoblastic or undifferentiated leukemia in children responds most favorably, but even in this disease where complete remissions occur frequently with disappearance of all detectable leukemic cells from the blood and marrow (702 to 705), the leukemic cells ultimately become resistant and the disease recurs.

Large doses of corticosteroids have manifold profound effects on normal tissues, which are classically described as the Cushing syndrome in man. In addition to their well-known effects on salt and water balance, intermediary metabolism, and connective tissue (675, 706, 707), they produce atrophy of thymic and other lymphoid tissue, lymphopenia, eosinopenia, and neutrophilia (708 to 714). Corticosteroids may exert a direct lymphocytolytic effect, as well as suppression of mitosis of lymphocytes (710, 715, 716, 717). Following the exposure of lymphocytes to adrenal steroids *in vitro*, destructive changes become evident within fifteen minutes, consisting of budding and shedding of cytoplasm and nuclear pyknosis and karyorrhexis (710).

A steroid hormone is presumed to act on responsive normal and neoplastic tissue in an identical manner. Attempts to alter the steroid molecules in order to dissociate the undesirable effects on responsive normal tissues (e.g. masculinization) from inhibitory effects on cancer cells have not been successful (680, 718 to 720). It is not known whether the diverse changes occurring in responsive cells from the regulatory influence of a particular steroid are due to multiple "primary reactions" at different sites in the cell, or to a single "primary reaction" which triggers a "propagated disturbance" which spreads through the cell, secondarily affecting many metabolic activities (721).

Following administration of labelled steroids, selective concentration of androgens has been found in male accessory sex tissues (722 to 724), and estradiol, and estrone to a lesser extent, are concentrated and retained in the rat uterus (725, 726). A greater percentage of free androgens or estrogen was recovered in the target tissues than in the liver. In the latter organ, the largest portion of radiometabolites consisted of conjugated or unidentified fractions, presumably representing biologically inactive degradation products (722, 725). Following administration of cortisol 4-C^{14} to adrenalectomized rats, only small amounts of labelled cortisol or its metabolites were found in the lymphatic tissue, and this had largely disappeared before degenerative morphologic changes in the lymphocytes were seen or before their production was inhibited (727).

Administration of androgens to animals causes an overall protein anabolic effect with nitrogen retention (652, 728). Accelerated protein synthesis has been demonstrated in a number of organs and tissues including accessory sex tissues (724, 729, 730), kidney (731, 732), and regenerating liver (733). It has variously been proposed that testosterone acts to increase protein synthesis by accelerating the peptide bonding of tRNA-amino acids (729),

by increasing the production of "nuclear" or "microsomal" RNA (730), or that it regulates the levels of mRNA which is attached to the ribosomes (734). Although estrogens do not cause as much nitrogen retention as androgens, striking growth of the uterus and other female sex tissues occur, and increased synthesis of protein, RNA and phospholipid has been demonstrated in the uterus (735). Following administration of estradiol to immature hens, increased synthesis of protein occurred in cell-free oviduct preparations; it was suggested that this is due to enhanced peptide-bonding of amino acids on the microsomes (729). Addition of steroid to cell-free preparation *in vitro* had no effect on protein synthesis (734, 735).

Steroids have been shown to catalyze the hydrogen transfer between reduced triphosphopyridine nucleotide (TPHN), and oxidized diphosphopyridine nucleotide (DPN) (736, 737). This may serve to couple TPNH generating systems to bound DPN in the mitochondrial electron transport system (738). Accumulation of DPNH enhances oxidative phosphorylation, leading to increased synthesis of adenosine triphosphate (ATP) (738). By stimulating transhydrogenase activity (739), a steroid could thus increase formation of high energy phosphate to facilitate synthetic processes in the responsive cell.

A 3α-hydroxy-steroid-mediated transhydrogenase has been found in rat liver and prostate which is sensitive to androgens, but not to estrogens (740 to 742). Testosterone does not itself facilitate hydrogen transfer in this reaction, but on the contrary inhibits it (743). However, androsterone, a metabolic conversion product of testosterone (744, 745) (Fig. 9), is a 3α-hydroxy-steroid which can initate this transhydrogenation reaction. It has been proposed that the anabolic effects of testosterone *in vivo* may be a result of a favorable balance between the (antagonistic) effects of testosterone and androsterone. Testosterone, by inhibiting transhydrogenation, may cause accumulation of TPNH which is required for biosynthetic processes. Depending on the availability of Δ^4-steroid hydrogenase for conversion of testosterone to androsterone (744, 745), the latter may accelerate hydrogen transfer and increase ATP formation. ATP, in a reaction catalyzed by nucleoside diphosphokinase, may then transfer phosphate to guanosine diphosphate (GDP) to form guanosine triphosphate (GTP). The latter co-factor is essential for peptide bonding (746), and it has been suggested that the availability of GTP may be a rate-limiting factor in protein synthesis (729).

An estrogen-mediated transhydrogenase has been found in human placenta, mammary tissues and breast cancer that is sensitive to 17-β-hydroxysteroids (e.g. estadiol-17β) (736, 737, 747, 748). It has been proposed that estrogens may influence biosynthetic processes by regulating a balance between TPNH and ATP in a manner similar to that proposed for androgen-mediated transhydrogenases.

While certain steroids can accelerate hydrogen transfer and increase

ATP formation, others inhibit DPNH oxidation and DPNH-cytochrome reductase activity (749). Such inhibition of electron transport could decrease metabolic activity in tissues inhibited by a specific steroid. The steroid-mediated transhydrogenation demonstrated in *in vitro* studies is an important mechanism, but has not yet been shown to play a significant role in the action of steroids under physiological conditions *in vivo*.

There are conflicting reports on the effect of estrogen on the permeability of uterine cells to sugar and amino acids (721, 750 to 752). Given impetus by the demonstration of the marked effect of insulin on cell permeability (753 to 755), a number of theories have been proposed as to how steroids might affect permeability of cellular membranes, including possible effects on diffusion barriers between intracellular compartments (i.e. endoplasmic reticulum or mitochondria), as well as effects on the cell wall. These theories have been summarized in several reviews (675, 721, 735, 756), and it is not known if steroids act on cellular membranes directly, or whether they influence permeability by secondary effects on certain enzymes on which the membrane transport systems are dependent.

Inhibition of oxidative metabolism by corticosteroids has been demonstrated in rat-liver mitochondria. Oxidative phosphorylation was not affected (757). Gallagher has proposed that this inhibition is secondary to a direct effect of hydrocortisone on the mitochondrial membrane, resulting in a decrease in its selective semi-permeability, and loss of soluble respiratory co-factors from the mitochondrial enzyme systems. White, on the other hand, has presented evidence that corticosteroid-induced inhibition of oxygen consumption and glucose oxidation as well as of protein and nuclei acid synthesis in lymphocytes is secondary to a release of latent ATPase activity in the mitochondria, thus decreasing the supply of ATP necessary for the cell's synthetic processes (758). As explanation for the situation in which a steroid might stimulate growth, Jensen has proposed conversely that estrogen might inhibit a nucleotidase (?ATPase) which is pre-existent in sensitive tissues, thereby increasing availability of high energy phosphates for biosynthetic processes, and active transport of substrates across membrane barriers (735).

Deoxycorticosterone (DOC), progesterone and a variety of other steroids added *in vitro* have been found to release latent ATPase activity of freshly prepared lymphosarcoma and liver mitochondria (759). Swelling of the mitochondria was observed after addition of DOC which could then be reversed by addition of ATP; simultaneous addition of $Mg^{++}$with the ATP resulted in activation of ATPase and negated the effectiveness of ATP in causing volume contraction. Thyroxine, as well as a number of other physiological and non-physiological substances, also cause mitochondrial swelling that can be specifically reversed by ATP (760). Lehninger relates this effect to loss of bound DPN and "uncoupling" or rather "loosening" of the coupling that normally exists between the transport of electrons down the respiratory enzyme chain (which is necessary for swelling) and the phosphorylation process which produces ATP (760).

Steroid hormones have recently been shown to cause glutamic dehydrogenase (GDH), an enzyme with a molecular weight of about 1,000,000, to disaggregate into four apparently identical sub-units with molecular weights of 250,000 (761, 762). While GDH is only active in catalyzing the amination of α-ketoglutaric acid to form L-glutamate in its most highly aggregate state (762, 763), the sub-units are more active than the aggregate in catalyzing the alanine dehydrogenase reaction. Estrogens and progesterone are most active in inhibiting GDH, while testosterone and adrenal corticoids are less so. DPN or ADP (763) and leucine and several other amino acids (762) favor aggregation of GDH and can prevent steroid (or DPNH) induced dissociation. The influence of steroids and these other biological materials on the physical state of the enzyme might provide a basis for regulation of its activity. Although this effect of steroids has as yet only been demonstrated with GDH, it has been proposed that similar mechanisms might operate in the regulation of other enzyme systems (762). Such a regulatory influence on an enzyme by a steroid which is itself structurally independent (i.e. not a steric analogue) of the enzyme may be an example of so-called "allosteric" regulation (764). In this regard, it should be noted that a number of non-steroidal estrogens have been synthesized (Fig. 9) which are active *in vivo* as well as in certain *in vitro* enzyme studies (735, 761, 765).

Considerable physiochemical information is available involving interactions between steroids and nucleotides (766 to 768), proteins (769, 770), and water-oil interfaces (771). Apparently, no chemical reaction is involved in these interactions (772), but the association of the steroid with the receptor portion of enzymes and other molecules may prevent interaction of the latter with other materials, thereby exerting a regulatory effect on cellular function (675, 725). Studies have been made on the conformation and location of polar groups of various steroids and of positional factors concerned with their physical mode of attachment to the receptors (772 to 776). But it has not yet been possible to correlate the results of those studies with specific physiologic steroid-induced responses in target cells.

It may be concluded that the steroid hormones may act on responsive cells to activate or suppress their growth by affecting several possible mechanisms: peptide bonding in protein synthesis; RNA production; TPNH; DPNH; ATP, and GTP levels; ATPase activity; state of aggregation of intracellular enzymes; and permeability of the cell membrane or of subcellular organelles. The structural specificities of the steroids in relation to their effects on certain tissues indicate that more precise answers may be soon available, but the exact mechanism of steroid action on cells has not yet been established.

CONCLUSION

Current views concerning the operation of a model cell have been described, and we have attempted to localize the action of various anticancer drugs in altering or damaging the machinery of the cell. A number of potent

and specific drugs are now available which act at defined points on cell structures or metabolic pathways, generally to produce cellular injury and death. With the availability of new and precise techniques, knowledge in this area will continue to accumulate rapidly.

It is hoped that drugs can be found which will have a more selective toxic effect on neoplastic cells. This may result from the discovery of qualitatively distinct pathways or structures in the neoplastic cell, or from the exploitation of quantitative differences between normal and cancer cells. In regard to the latter possibility, the advantages of combining drugs with known mechanisms of action to magnify a minor therapeutic advantage requires more attention. The development of resistance to specific drugs by cancer cells poses a formidable problem. Further accumulation in our knowlege of the precise action of new drugs in influencing cell growth will allow us to treat the ever present and pressing problem of disseminated cancer far more effectively.

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