**Abstract.** Alkylating agents, for example nitrogen "mustards", are variably toxic, mutagenic, carcinogenic and teratogenic, but by mechanisms which have not been clearly established. In particular, the mechanisms both of their delayed toxic effects (which are primarily against dividing cells, in association with retardation of the rate of cell division, disruption of mitoses, and breakages and other abnormalities of chromosomes) and of their carcinogenic actions are not understood. The literature on the testing of thousands of analogues has demonstrated great variability of effects on the various cell biological phenomena, and no aspect of chemical structure or biochemical reactivity of these agents has been established as especially related to any particular effect. Here theories of the mechanisms of action of alkylating agents are reviewed and it is suggested that impairment of the functions of DNA polymerase complexes might contribute to some of the effects of alkylating agents. In particular, impairment of replicative fidelity of DNA during the S-phase could contribute to some of the mitotic and chromosomal effects, as well as to their carcinogenic and teratogenic potencies. Some aspects of testing the effects of alkylating agents on components of the DNA synthetic pathway are mentioned. Emphasis is given to consideration of the various relevant levels (conventional plasma/tissue; tissue/tumour cell cytoplasm; tumour cell cytoplasm/tumour cell nucleus and tumour nuclear karyoplasm/tumour chromatin) of the pharmacokinetics and pharmacodynamics of the agents and their metabolites.

"Mustard gas" and its nitrogen analogues

Following its use as a poison gas in World War I, beginning in 1917 on the Western Front (1), "mustard gas" [the alkylating agent bis(2-chloroethyl)sulphide] was recognised to cause not only acute toxic effects on exposed tissues, but a variety of delayed systemic disorders including leukopenia and gastrointestinal ulceration among the affected soldiers (2, 3). In the 1930s, nitrogen analogues ("nitrogen mustards") of this compound were synthesised and found to be more rapidly absorbed through the skin than sulphur mustards (4). Because this increased absorption meant potentially greater systemic effects for soldiers if exposed to such agents in combat (4), the biological and biochemical effects of both types of agents, especially in low doses, were investigated in secret during World War II and the results were made public only after 1945 (4). The delayed biological effects of low doses of these agents were found to be mainly against dividing cells (3, 4), causing, in particular, inhibition of cell division generally; abnormalities of mitotic morphology (lesions of the spindle and abnormal chromosomal segregations) and chromosomal lesions (increased breakages, mis-sited reunions, "stickiness" and deletions). In additional work, alkylating agents were found to cause germline mutagenesis (5); carcinogenesis (6) and teratogenesis (7) (in so far as cells of normal embryos are more susceptible to these agents than are the normal cells of the mother animal).

The biochemical actions of alkylating agents were found to depend on preliminary ionisation of the parent compound, at subsequent reaction of the positively-charged base with negatively-charged groups on tissue macromolecules. Reactions of the bases with proteins and the consequent effects on enzyme activities were investigated first (3). Alkylating agents were found to react particularly avidly with cysteinyl derivatives, as well as with alpha carbonyl, aspartyl, glutamyl and imidaxole (histidine) groups at physiological pH and low concentrations (8). The corresponding enzymic and cytoplasmic structural damage appeared to account for the acute toxic effects of the agents.

However, the delayed systemic effects of these agents were found to occur at much lower doses (e.g., 1 mg/kg) (9) than are required for acute effects and, in the case of carcinogenesis particularly, to occur long after administration. This implied that the mechanism(s) of these effects must involve some on-going, and possibly self-perpetuating, damage...
to the cell. With the discovery of the structure of DNA and its role in cell heredity, the reactions of alkylating agents with this macromolecule were studied. It was found that such reactions occurred at many sites and, perhaps especially, with the phosphate-ester bonds of the "backbone" of the molecule, as well as with the N7 position of guanine (8).

In the same period (late 1940s-1950s), investigations of the effects of chemical analogues of the dichloroethyl mustards were undertaken in the hope of finding drugs with less systemic toxicity and more anti-mitotic specificity. In this way, chlorambucil and Myleran were developed, especially by Haddow and others at the Chester Beatty Institute in London (2). Analogues with two ionisable chlorine atoms ("bi-functional" agents) are usually more potent than those with only one such group ("mono-functional" agents) (2, 4). This particular finding was thought to indicate that the mechanism of action involved cross-linking by the agent between adjacent reactive groups of macromolecules, either between proteins (10), between strands of DNA (11), or even possibly between nucleic acids and proteins (12).

**Variability of the biological effects of alkylating agents.** Detailed investigations of the cell biological effects of alkylating agents followed, showing first that some alkylating agents can reduce DNA synthesis, without affecting RNA synthesis, despite the template for both enzyme systems being the same (13-15). Further, a wide variety of actions of alkylating agents were found in various types of comparative studies, as detailed in the following subsections.

**Variability of particular biological effects according to species or experimental preparations.** Initially, variance in biological effects became apparent related to the species used or other aspects of the experimental preparation. This became especially evident during the attempts to establish whether it is the rate of synthesis of DNA on the one hand, or the integrity of the mitotic process or chromosomes on the other, which is the primary cell biological "target" of alkylating agents. Early studies by Auerbach, Darlington and others in the 1940s (reviewed 3), using plant cells, indicated that the doses of alkylating agents which are able to inhibit the rate of mitosis (taken as an indication of the rate of DNA synthesis) were lower than those necessary to disrupt chromosomes. Nevertheless, Koller (16) found the opposite, in that the lowest doses of an alkylating agent (CB 2041), which caused mitotic and chromosomal lesions in Walker 256 rat carcinoma cells, did so without detectable temporary or permanent inhibition of cell growth. (For other contributions to this controversy, especially in relation to effects on plant cells, see 17, 18).

Later work concerned with the same issue was directed to investigations into the sensitivity of cells according to the phase of the cell cycle during which the alkylating agents were applied. Thus, whether or not cells are damaged during the synthetic ("S")-phase, or are damaged in either or both of the resting ("G")-phases, relates to DNA synthesis as the "target" of the agents. Some anticancer drugs affect target cells more in one phase than another of the cell cycle (19, 20), and it has been suggested that alkylating agents are particularly active in G2 ("G2 arrest", 21). However, later work, summarised by Meyn and Murray (22), showed that the most sensitive phase of the cell cycle can vary between alkylating agents and, furthermore, for a given drug, can vary according to the cell line used for the study. The currently most widely-accepted view is that the actions of alkylating agents do not involve any particular phase of the cell cycle (19, 23).

**Variability of biological effects among chemical analogues and according to "target" tumour type.** Haddow and co-workers (24) reported great variability of the biological effects among analogues of alkylating agents and according to the "target" tumour types. In a later detailed study, Koller (16) documented the effects of eighteen different analogues of mustard gas on "temporary" and "permanent" growth inhibition of the same cell line, as well as on three different cytotoxic effects: mitotic suppression; chromosomal breaks and bridges at 48 hours (which he termed "radiomimetic effects"); and excessive fragmentation with loss of relationship to the spindle apparatus at 72 hours (which he termed "cytotoxic effects"). No consistent patterns among the eighteen analogues were found and, furthermore, a different spectra of effects were obtained when the same tests using the same analogues were applied to other carcinoma cell lines.

Similar variation concerning the comparative effects of alkylating agents were obtained by Németh and co-workers (25) and Stock and Sigura (26), the latter authors concluding that different tumours have such markedly different susceptibilities to the various alkylating agents that no single experimental model was sufficient for the evaluation of these agents for human use.

**Variability of the effects of the same agent on different biological phenomena.** Yet another type of study to reveal the enormous differences in the effects of alkylating agents were the comparative studies attempting to correlate the anti-neoplastic, mutagenic and carcinogenic potencies exhibited by various classes of agents.

Walpole (6) found, in relation to ethylenimines, that: "... neither polyfunctionarity nor cross-linkage, nor the formation of a polyreactive polymer or micelle is necessary for carcinogenic action in ethylenimine derivatives, whatever may be their significance for other radiomimetic effects."

Strauss and co-workers (27) noted the literature indicating that bi-functional agents are, one the one hand,
more toxic and, on the other, less mutagenic than mono-functional agents. To complicate the matter further, these authors found that the relative ratios of carcinogenicity to mutagenicity with respect to bi- versus mono-functionality varied according to the type of agent. Citing one example, Strauss and co-workers (27) pointed out that: "...NTG (N'-nitro-N-nitrosoguanidine) is remarkable among mutagens for its mutagenic efficiency at low lethality. Some factor other than the methylation of bases must determine the ratio between mutagenic effect and the toxicity of monofunctional alkylating agents."

Magee (28) studied the toxic, carcinogenic, mutagenic and teratogenic effects of various alkyl nitrosamines and could identify no structural features to correlate with these biological effects. He even suggested that: "...alkylation is far from being established as the critical intracellular reaction responsible for the biological activity of the nitroso compounds."

Van Duuren (29) reviewed the biological effects of epoxides, lactones and halo-ethers and, citing earlier work, concluded that the potencies of the carcinogenicity, mutagenicity and tumour-initiating activity of twenty-two agents, including alkylating agents, aromatic hydrocarbons and acridine dyes, were not in any way clearly correlated with each other.

Subsequent work. Later investigations (reviewed 22, 30-32) have contested none of these general conclusions, nor clarified their causes, nor found significantly less variability in studies of either human tumours, or human tumour cell lines in vitro. Meyn and Murray (22) stated: "Despite the passage of some 20 years since that (variability of effect) discovery, we still have no explanation of such variations". Similarly Michaelson (32), after reviewing the effects of alkylating agents, concluded that: "The mechanisms of cytotoxicity remain poorly understood."

Previous theories of the species-, cell type- and tumour type-variability of actions of alkylating agents

In the 1950s and 1960s, theories about the variability of the biological effects of alkylating agents were little discussed, except perhaps by those authors who were attempting to design anticancer drugs based on alkylation. The underlying idea of these attempts to design drugs appears to have been one of variable intra- or sub-cellular pharmacokinetics and -dynamics. Thus Bergel (33) indicated that rational drug design required knowledge of a) how carcinogenesis begins, b) every detail of cell and tissue composition, c) the difference between normal etc. material, and d) the exact nature of the interaction between the drug and all cellular constituents. (Bergel (34), observed that very few of these requirements were being met at that time). This field has been somewhat neglected, in that clinical pharmacokinetic and pharmacodynamic studies have tended to emphasise mainly the organ and tissue levels (34). This issue is considered further below under "Aspects of testing".

A second hypothesis has been that the key to the biological variability of drug effects is in the possible differential capacities of each tissue to generate active metabolites. This is a generally similar idea to that of activation of chemical carcinogens, which was demonstrated to be important in experimental hepatic carcinogenesis by Miller and Miller (35) in 1949, and reviewed by Lawley (36). To date, no variability of activation of alkylating agents, which might account for the variable cell biological effects, has been demonstrated in any extensive study.

A third hypothesis has been that variations of effectiveness of the de-alkylation (37) or of the repair of DNA varies on a species-, cell type- or tumour type-specific basis (38-40). Dealkylation occurs by transfer to an "alkyl-acceptor protein" [a "cleaning" or "mopping-up" protein, rather than a (properly defined) enzyme], although the larger alkyl groups are not removed by this mechanism (38). It has been considered, since the early 1990s, that mammalian O6-guanine-DNA methyl transferase plays an important role in carcinogenesis induced by alkylating agents, as well as in antitumour nitrosourea chemotherapy (41). In addition, the toxicity of the clinically utilised methylating agent temozolomide has been attributed to mismatch repair of O6-methylguanine (42). More recently, it has been described that the repair of N-methyl adducts by base excision repair is also an important determinant of temozolomide-mediated cytotoxicity (43). The applicability of these and other studies to the effects of other alkylating agents in various experimental and clinical systems is not yet fully established.

Impaired fidelity of replication of DNA and alkylating agents

From the above, it is apparent that the effects of alkylating agents in low doses are on cell processes in which DNA is involved and, such is the diversity of events caused by small changes of chemical structure between analogues, that many highly structure-sensitive sites may be the potential targets of these agents. The best-documented series of biochemical processes, which can have diverse outcomes and are dependent on a variety of structure-sensitive enzymic sites, are those associated with the synthesis of faithfully replicated DNA nucleotide sequences (44-49). Further, interference with these processes can either inhibit DNA synthesis or cause the newly-synthesised DNA strands to be unfaithful replicas of their templates. Potentially, the sites which could be responsible for either or both of these
changes are numerous and include the helicase, synthetic, proofreading and mismatch repair steps (44, 45). Several of these steps are known to be potentially susceptible to the actions both of endogenous and exogenous factors and thus capable, when deranged, of causing reduced fidelity of DNA replication (46-49). In particular, Kunkel (49) has shown that amino acid alterations at a distance from the DNA synthetic pocket can have this effect. This constitutes an indirect, albeit "endogenous", mechanism of action of factors which might impair the replicative fidelity of DNA and also, possibly, reduce the speed of DNA synthesis, which might well be imitated by exogenous chemicals (see below).

Differing types of genomic lesion according to different abnormalities of the amino acid sequence in the DNA polymerase complex. Much of our understanding of genomic lesions caused by abnormal DNA polymerase complexes and related enzymes has come from the investigation of inherited predispositions to tumours in humans. Thus, some of the human predispositions to colonic cancer are associated with germline mutations, which reduce the repair of "slippage" insertions or deletions of specific short nucleotide sequences within repetitive sequences of the particular sequence. One common example of this is changes of "n" in the repetitive sequence (cytosine-adenine)n (50, 51), which then is detectable by gel electrophoretic methods as "microsatellite instability". However, other inherited predispositions to tumours (52), for example, the Li-Fraumeni syndrome, are associated with infidelity of replication of DNA but do not cause such short-sequence insertions or deletions into repetitive sequences. The implication of this is that different inherited alterations of amino acid sequence in the DNA polymerase complex or related complexes may cause different distortions of the relevant active sites of the complexes and, as a consequence, can result in different types of genomic mutational lesion, with widely different, and possibly cell-type specific, phenotypic changes (50).

Replicative fidelity of DNA and alkylating agents. Although the importance of replicative fidelity of DNA was inherent in the biological role of DNA, as elaborated in the 1950s, the idea that reduced fidelity of replication of DNA might be an effect of alkylating agents appears to have been little considered. In original work on the timing of chromosomal abnormalities induced by alkylating agents, Darlington and Koller (53) noted that such effects continued in cells after the removal of the drug. However, spontaneous and thermally-induced chromosomal abnormalities were not followed by such later abnormalities. These authors (53) concluded that alkylating agents cause "injury to the nucleic acid metabolism" of cells. Alexander (18), in the course of arguing that reduction of DNA synthesis may not be the primary effect of alkylating agents, suggested the possibility of abnormality of DNA after subsequent treatment being the cause of reduced rates of synthesis: "A qualitative defect in the DNA made would be consistent with the posttreatment growth pattern, but not a quantitative interference with DNA synthesis."

Baril and co-workers (54), in 1975, showed that nitrosoureas can inhibit synthesis of DNA by DNA polymerase II, by acting directly on the enzyme rather than the DNA template. However, these authors did not investigate possible infidelity of the replication of DNA as an action of alkylating agents.

Auerbach (55), in 1976, briefly referred to infidelity of replication of DNA as a possible effect of alkylating agents, without substantial discussion. Lawley (36, 38) discussed the effects of alkylation specifically on DNA repair, but there has since been little additional discussion of DNA synthetic and related enzyme complexes as targets of alkylating agents.

Models involving DNA polymerases of the biological effects of alkylating agents

None of the foregoing studies provided definitive evidence for the role of DNA polymerases in the biological effects of alkylating agents. However, the biochemical parameters have been established according to which some suggestions for the roles of such actions may be made. This is based on the possibility that interference with DNA polymerase complexes might cause inhibition of DNA synthesis, or infidelity of replication of DNA, or both (Figures 1-4).

Rate of synthesis of DNA. Cross-linking between DNA strands by bi-functional alkylating agents can be accepted as a mechanism of inhibition or arrest of synthesis of DNA (Figure 1). If cross-linking between DNA and protein occurs, then such linking between DNA-adducts and other enzymic sites of DNA synthesis might also occur (Figure 1). Should the event of alkylation occur on a single strand of DNA after the action of a helicase, then synthetases, proofreading, mismatch repair and other related enzymic sites could be alternative locations which are susceptible to such actions (Figures 1, 4).

Mono-functional agents might cause inhibition of DNA by obstruction of the enzymic "pockets" at various sites, either by linking to the template DNA or to the enzymic sites themselves (Figure 2). An additional specific possibility is that these agents may effectively retard the movements of the relevant parts of the polymerase complex during addition of nucleotides in the "hinge" mechanism (49) of the synthetic site (Figure 3, 4). Mono-functional agents may be less potent than bi-functional agents because the former, as adducts, may be more easily removed by de-alkylation (by
Figure 1. Schematic representation of some of the possible sites in the DNA replicative process, where cross-linking actions of bi-functional alkylating agents could cause inhibition of the synthesis of DNA. (Not to scale). This diagram implies that bi-functional agents might also inhibit DNA synthesis by obstructive phenomena, indicated in Figure 2.

Figure 2. Schematic representation of some of the possible sites in the DNA replicative process, where obstructive actions of mono-functional alkylating agents could cause inhibition of the synthesis of DNA. (Not to scale).
the various DNA rectification mechanisms, see above). In particular, bi-functional adducts might reduce the accessibility of the dealkylating proteins to the sites of binding because of their cross-linking effects.

**G2 arrest.** In G2 arrest, progress from the S-phase to the M-phase is delayed. In this phase, condensation of DNA into chromosomes occurs in association with synthesis of proteins and RNA. There being no DNA synthesis in this phase, a role for reduction of DNA synthesis is unlikely. Therefore, the mechanism of G2 arrest by alkylating agents has been widely attributed to reduced synthesis of either protein or RNA (19).

A relatively unconsidered mechanism of G2 arrest is that the agent may have caused massive infidelity of DNA replication, and that extensive repair is necessary before condensation can occur, thus prolonging the G2-phase. In severe cases, G2 arrest may be followed by mitotic and chromosomal abnormalities, because the degree of infidelity of the new DNA is too great for the repair mechanisms to correct (18).

**Disruption of mitosis.** These abnormalities are thought to occur either because of deficiencies of the spindle fibres, or of the binding of the centromeres of the chromosomes to the spindle fibres. Alkylating agents are active molecules, but in low doses do not seem to have specific anti-tubulin actions (34). However, they might act on the kinetochore proteins (56) which form the binding sites for the centromeres. Alternatively, alkylating agents might cause such damage to the centromeres that they are unable to bind to their appropriate binding sites on the spindle fibre.

**Chromosomal abnormalities.** "Stickiness" of chromosomes (in terms of "chromatic connecting threads") was described by Hansemann (57) in the 1890s, and discussed in later reviews (16, 53, 58), but its mechanisms have not been studied in great detail (59). It is possible that incompletely condensed chromosomes might have "internal" material, exposed on the surface of the chromosomes, allowing for chromosome-to-chromosome binding. If this were so, then severe infidelity of replication of DNA could be a mechanism of this original incompleteness of condensation of the chromosome.
Chromosomal breakage necessarily involves rupture of the phospho-diester bond of the "backbone" of the DNA chain. This can occur as a direct effect of the action of alkylating agents (8) and, in turn, may be more likely to occur during the S-phase of the cell cycle, which is a time when the DNA chain is not encrusted with histones and, thus, possibly exposed to attack by cytoplasmic alkylating agents. Another factor involving this scenario could be that, because infidele DNA is kept in the "bare" state for longer owing to the prolongation of the phase of DNA-cleansing and other repairs, the "backbone" is therefore exposed for longer to breakage by alkylating agents. Some support for this concept comes from the documentation of decreased stability of DNA in cells treated with alkylating agents (60).

**Germline "point" mutations.** These may occur simply by replicative infidelity occurring during meiosis, as discussed in reference (61).

**Induction of tumours by alkylating agents.** In previous papers (62-64), the present author discussed the possibility that many chemical carcinogens may cause tumour formation by acting on DNA polymerase complexes, either via the induction of genetic instability as a first step, or by more particular mutation of the genomes of local stem cells. It is proposed here that alkylating agents may act in a similar "nongenotoxic" way, by disturbing the three-dimensional structure of the "pockets" of the relevant enzymic sites (Figure 3).

**Teratogenesis.** Embryonal cells have more insertion sites for DNA polymerases than adult cells (65), and thus presumably more DNA polymerase complexes acting per cell during the S-phase. Both because of this relatively higher concentration of DNA polymerase complexes in mitotic embryonal cells than in mitotic adult cells, and also because embryonal cells may carry out more mitoses than adult cells during the same period of time of exposure to the agent, embryonal cells may be more susceptible to the action of alkylating agents.

**DNA synthesis vs. RNA synthesis.** The fact that some alkylating agents impair DNA synthesis without affecting...
RNA synthesis (13-15) may be explicable if the agent affects the DNA polymerase complex but not the RNA polymerase complex.

Variability of biological effects according to species, tissues and tumours types. Since the 1950s, it has been considered that the explanations of these variations might lie in the variable pharmacokinetics and pharmacodynamics which may apply to these agents according to species, and also (at least by Bergel, 33) according to tissue type, or even tumour type (see above). However, it is further possible that a component of the variability could be due to species susceptibility of DNA polymerases to injury by alkylating agents. However, there is little literature on the comparative effects of alkylating agents on the replicative fidelities of DNA polymerases between mammalian species.

Aspects of tests of alkylating agents in relation to the enzymes of synthesis of DNA

The recently accumulated information and methodology concerning the roles of particular proteins and other molecules in cell biological processes indicate new achievable objectives for testing the biological effects of these agents. It is not possible here to detail all the potentially relevant experiments which might be conducted using the new techniques. It could be noted, however, how clearly the literature has established that recovery of a drug or its metabolite from a particular biochemical "site" in association with a particular cell biological effect does not prove causation, but is a helpful first step.

It is worth noting that the effects of alkylating agents could be tested on the functional capacities of these newly-discovered molecules in cell-free, cell culture and possibly in in vivo experimental systems. In such experiments, however, a major consideration may be the subcellular pharmacokinetics and pharmacodynamics of the agents. This area of investigation has been relatively neglected (66), although its importance was signalled by the early designers of alkylating anticancer drugs (see Bergel, 33 and above). Particular aspects of these possible subcellular events have been noted above, viz, the activation of parent agents to active metabolites, and the "mopping up" especially of adducts, by the intranuclear enzymes of the affected cells. However, factors such as degradation or sequestration of either the parent drug or its activated metabolite by any macromolecule within the site of administration or the chromatin of the tumour cell nuclei may also be relevant. That is to say, in the investigation of therapeutic aspects of these mechanisms, pharmacodynamics and pharmacokinetics must be considered at all the various sequential levels which are now demonstrable by contemporary cell biological techniques. These levels include not only the conventional one between plasma and tissues, but also between normal tissue and tumour cell cytoplasm, between tumour cell cytoplasm and tumour nucleus, as well as that between the karyoplasm of the tumour cell nucleus and the tumour nuclear chromatin.

A second aspect is that the use of cultured cell lines may not provide sufficiently appropriate models of tumour cells in the human patient, because many widely-available cell lines are "transformed" to some degree and may be genetically unstable (67), thus affecting their subcellular pharmacokinetic and -dynamic repertoires.

In relation to testing for carcinogenic effects of alkylating agents, the experiments suggested by the present author (63) may be useful.

Conclusion

Alkylating agents have been widely used as therapeutic agents since the 1950s but, despite many studies and the availability of large numbers of analogues, the significant biochemical and cell biological "target(s)" of these agents are still unknown, and no completely satisfactory experimental test is available for assessing either the cytotoxic efficacies or the carcinogenic potencies of new agents. This pharmacological hiatus has probably inhibited the exploration of the therapeutic value of these agents in specific clinical situations. The combination of the recent discoveries concerning the extreme dependency of fidelity of replication of DNA on the structures of the various enzymic sites of DNA polymerase complexes, and the previously documented marked variation of the actions of analogues of alkylating agents according to small chemical structural differences, suggest that the actions of the latter on the former may warrant detailed investigation.

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