# STUDIES ON BIOCHEMICAL AND PHARMACOLOGICAL EFFECTS OF CYCLOPHOSPHAMIDE AND PHOSPHORODIAMIDIC ACID

# STUDIES ON BIOCHEMICAL AND PHARMACOLOGICAL EFFECTS OF CYCLOPHOSPHAMIDE AND PHOSPHORODIAMIDIC ACID

By

YVONNE H. ADIWINATA SURYA, B.Sc.

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AUTHOR: Yvonne H. Adiwinata Surya, B.Sc. (Simon Fraser University)

SUPERVISOR: Dr. B.L. Hillcoat, Professor, Department of Biochemistry

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SCOPE AND CONTENTS: Recent evidence suggests that phosphorodiamidic acid is formed from cyclophosphamide *in vivo* and *in vitro* and may be the active form of this drug.

We have studied the effect of phosphorodiamidic acid and cyclophosphamide on the growth of the mouse leukemic cell lines LM4 and LS2 in culture. On this basis, phosphorodiamidic acid was at least 100 times more potent than cyclophosphamide in inhibiting growth. It also produced enlargement of cells, an effect not seen with cyclophosphamide.

Although two metabolites of cyclophosphamide, phosphorodiamidic acid and acrolein were isolated and identified, attempts to measure these metabolites accurately were unsuccessful. Consequently the stoichiometry of the conversion could not be determined.

Phosphorodiamidic acid significantly increased the amount of cross-linked DNA after incubation with intact LM4 cells or isolated nuclei from these cells. Cyclophosphamide had a similar effect only in the isolated nuclei.

These findings strengthen the proposed role of phosphorodiamidic acid as the active metabolite of cyclophosphamide.

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## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| RNA | = | ribonucleic acid |
| DNA | = | deoxyribonucleic acid |
| NBP | = | 4-p-nitrobenzylpyridine |
| CP | = | cyclophosphamide |
| PD | = | phosphorodiamidic acid |
| $NADP^+$ | = | nicotinamide adenine dinucleotide phosphate (oxidized form) |
| NADPH | = | nicotinamide adenine dinucleotide phosphate (reduced form) |
| TLC | = | thin layer chromatography |
| GC-MS | = | gas chromatography - mass spectrometry |

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# 1. INTRODUCTION

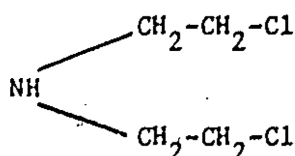
## 1.1. Chemical properties and biological action of alkylating agents

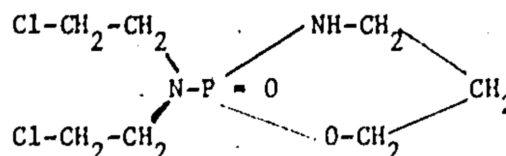
Alkylating agents are highly reactive chemical compounds capable of substituting an alkyl group (ie. $R\text{-}CH_2\text{-}CH_2^+$) for hydrogen atoms of many organic compounds. There are two classes of alkylating agents, monofunctional and polyfunctional. Monofunctional alkylating agents have only one active alkyl group, while the polyfunctional alkylating agents have two or more. In general, the polyfunctional agents have greater antitumor activity than the monofunctional (Cline and Haskell, 1975).

The differences in activity among the various alkylating agents apparently relate to differences in absorption, rate of metabolism and tissue affinity rather than to a basic difference in their mode of action (Cline and Haskell, 1975). All undergo strongly electrophilic chemical reactions through the formation of carbonium ion intermediates or of transition complexes with the target molecules. Hence there are two generally accepted basic mechanisms of alkylation, first order nucleophilic substitution ($Sn_1$) and second order nucleophilic substitution ($Sn_2$) (Warwick, 1963). Many groups such as the phosphate, amino, sulfhydryl, hydroxyl, carboxyl and imidazole groups present in biological molecules can be alkylated and several studies suggest that the reaction of alkylating agents with DNA relates to their cytotoxic effects (Van Durn, 1969; Wheeler, 1973 and Calabresi and Parks, 1976).

1.2. Toxicological properties of cyclophosphamide

Hundreds of compounds bearing potential alkylating groups have been synthesized and evaluated in an effort to obtain greater specificity against malignant as compared to normal tissues. Alkylating agents such as bis-(2 chloroethyl)-amine (A) are toxic to both normal and tumor tissues. Friedman and Seligman (1954) made a series of phosphamide derivatives of (A) in an attempt to lower the toxicity of the compound while its chemotherapeutic potency was preserved. Arnold and Bourseaux (1958) synthesized cyclophosphamide, 2-[bis-(2-chloroethyl)-amino]-2H-1,3,2,oxazaphosphorine,2-oxide, which has little cytotoxic or alkylating activity in vitro, and which requires activation by an enzyme of liver microsomes (Cohen and Jao, 1970).




(A) bis-(2-chloroethyl)-amine (B) cyclophosphamide

Cyclophosphamide is an effective antitumor agent by both oral and intravenous routes of administration. It is moderately soluble in water and can be readily extracted from aqueous solution into lipid solvents.

1.3. Metabolic conversion of cyclophosphamide

1.3.1. Microsomal activation in vitro: The nature of activation of cyclophosphamide whereby it exerts its cytotoxic action has been extensively investigated. Arnold and Bourseaux (1958) designed cyclophosphamide, hoping that tumor cells may contain a phosphorodiamidase

enzyme to hydrolyze the P-N linkage, thus liberating a reactive alkylating species. However, activation has been shown to occur primarily in the liver rather than in the tumor tissues.

Liver homogenates (Foley et al, 1961) or isolated liver microsomes (Cohen and Jao, 1970; Connors et al, 1970) when supplied with reduced nicotinamide adenine dinucleotide phosphate and oxygen in vitro, will transform cyclophosphamide to alkylating and cytotoxic metabolites. Sladek (1971) studied extensively the enzymatic conversion of cyclophosphamide to alkylating metabolites and found this enzymatic activity localized in the liver microsomal fraction.

The initial oxidative step in cyclophosphamide metabolism is due to the microsomal mixed-function oxidase system. Sladek (1972) and Ohira et al, (1975) showed that pretreatment with agents which stimulated or inhibited the rate of production of hepatic microsomal mixed-function oxidase markedly influenced the metabolic conversion of cyclophosphamide to active alkylating agents. The microsomal mixed-function oxidase system converted cyclophosphamide into 4-hydroxycyclophosphamide. Direct evidence for generation of 4-hydroxycyclophosphamide in vitro has been provided recently by Connors et al, (1974). Sladek (1973) reported evidence demonstrating the generation of an alkylating aldehyde from cyclophosphamide in vivo and in vitro in biological and chemical systems, and Struck (1974) has positively characterized aldophosphamide as its semicarbazone derivative. The compounds, 2-carboxyethyl-N,N,bis-(2-chloroethyl)-phosphorodiamidate (or carboxycyclophosphamide) and 4-ketocyclophosphamide have been identified as metabolic products of

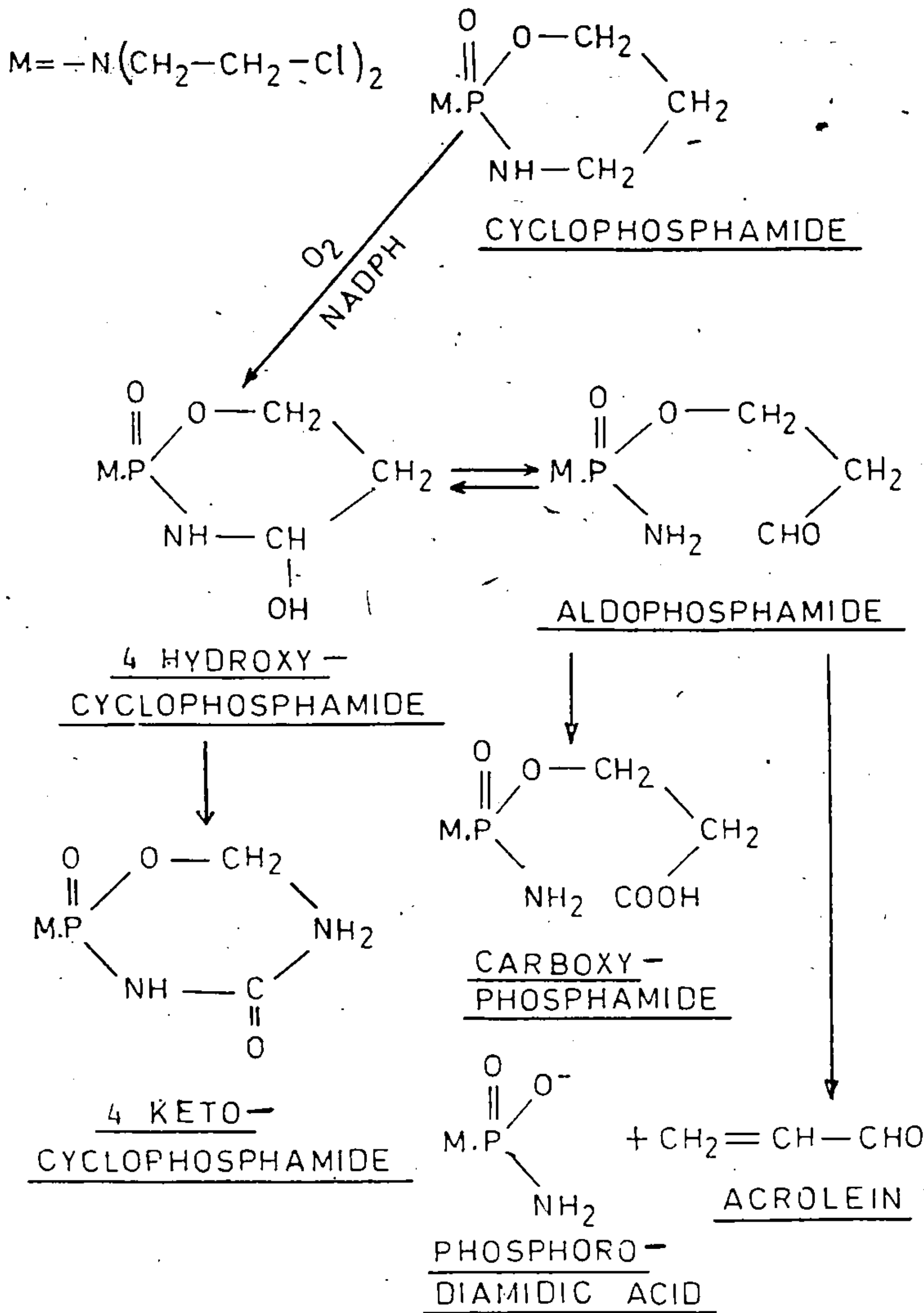
cyclophosphamide in men and dogs (Struck et al, 1971). Alarcon and Meinhofer (1971) identified acrolein as a product of the microsomal incubation of cyclophosphamide and N,N-bis(2-chloroethyl)-phosphorodiamidate (or phosphorodiamidic acid) has also been identified both in vitro (Colvin et al, 1973; Connors et al, 1974) and in vivo (Struck et al, 1975; Fenselau et al, 1975). From the evidence available, a series of biotransformation reactions was postulated as shown in Fig. 1.

1.3.2. Identification of active metabolites: The main difficulty in clarifying the metabolism of cyclophosphamide was the assay, isolation and identification of the metabolites as well as their synthesis. Only after a sufficient quantity of the various metabolites was available in pure form did the pharmacologic characterization and evaluation become possible.

Because of their instability, special efforts were necessary for the chemical synthesis of the primary activated metabolites, the tautomeric compounds 4-hydroxycyclophosphamide and aldophosphamide. Takamizawa et al, (1973) succeeded in synthesizing 4-hydroperoxycyclophosphamide, the reduction of which permitted the preparation of 4-hydroxycyclophosphamide. Friedman et al, (1963) synthesized phosphorodiamidic acid but aldophosphamide has not yet been obtained in a pure form.

Cyclophosphamide and its metabolites are characterized by determining their chemical and biological activities in vitro and their pharmacotherapeutic properties in vivo. Their chemical reactivity can

Figure 1: Scheme of metabolic activation of cyclophosphamide from Connors *et al* (1974b).


M= —N(CH2—CH2—Cl)2
CYCLOPHOSPHAMIDE
O2
NADPH
4 HYDROXY—
CYCLOPHOSPHAMIDE
ALDOPHOSPHAMIDE
4 KETO—
CYCLOPHOSPHAMIDE
CARBOXY—
PHOSPHAMIDE
PHOSPHORO—
DIAMIDIC ACID
+ CH2=CH—CHO
ACROLEIN

be assessed by means of the [4-(p-nitrobenzyl)] pyridine assay, and by their behavior in aqueous solution where the rate of liberation of chloride ion from the 2-chloroethyl group is taken as a measure for chemical reactivity. Their cytotoxic potency is assessed by comparing the concentration which kill 50% of cancer cells after incubation in cell culture (EC 50). Their therapeutic effect in vivo is measured by determining the $LD_{50}/CD_{50}$ ratio (50% lethal dose/50% curative dose) against tumor cells in intact animals. Table 1 contains data of chemical reactivity and biological activity of cyclophosphamide metabolites and nitrogen mustards compounds (Brock, 1976). Preliminary studies by Maddock et al, (1966) and Connors et al, (1974b) indicate that phosphorodiamidic acid is an active metabolite. Phosphorodiamidic acid has been shown to have higher toxicity at much lower concentrations than cyclophosphamide. Table 1 shows that phosphorodiamidic acid indeed has intense alkylating activity but its cytotoxic activity in only 2.5 CU/μmole. One cytotoxic unit (CU) is that amount of a cytotoxic compound which produces survival of 50% of animals after inoculation with tumor cells. On the other hand, 4-hydroxycyclophosphamide had high cytotoxic activity, 63 CU/mole, and showed a similar $CD_{50}$ index to cyclophosphamide, but weak alkylating activity. Voelcker et al, (1974) showed that only after cleavage of the phosphoric ester bond of 4-hydroxycyclophosphamide to form phosphorodiamidic acid and acrolein, that it was strongly alkylating. The 4-hydroxy derivative of 5,5 dimethylcyclophosphamide has been isolated (Cox et al, 1976a) and has low toxicity to both normal and tumor tissue. This compound cannot release phosphorodiamidic acid by β-elimination due to the gemdimethyl group at $C_5$, suggesting the importance of the release of phosphorodiamidic acid and acrolein during the activation step.

Table 1. Chemical reactivity and biologic activity of nitrogen mustard compounds and CP metabolites.

| Nitrogen mustard derivatives | Molecular weight | In vitro | | | | In vivo | | |
|---|---|---|---|---|---|---|---|---|
| | | Alkylating activity* (%) | Chloride ion hydrolysis: 1 val/mol† in | Cytotoxic activity‡ (CU/μmol) | CD50‡ (mg/kg) | LD50 (mg/kg) | D50 index (LD50/CD50) | |
| CP | 279§ | 1.3 | > 7 days | < 0.03 | 1.25 | 220 | 175 | |
| 4-Keto-CP | 275.1 | 1.2 | > 7 days | < 0.07 | > 800 | > 800 | - | |
| 4-Hydroxy-CP | 277.1 | 65 | 480 mins | 63 | 1.25 | 150 | 120 | |
| 4-Hydroperoxy-CP | 293 | 40 | 240 mins | 21 | 1.25 | 97.5 | 78 | |
| Carboxyphosphamide | 293 | 85 | > 7 days | 0.1 | 200 | 800 | 4 | |
| N,N-Bis-(2-chloroethyl)phosphorodiamidic acid | 221 | 90 | 60 mins | 2.5 | 20 | 61 | 3.5 | |
| Acrolein | 56.06 | (1.9) | - | 0.4 | > 2.15 | 7.3 | - | |
| Nor-nitrogen mustard | 178.5 | 100 | 60 mins | 1.35 | 40 | 100 | 2.5 | |
| Chlormethine | 192.5 | 100 | 15 mins | 138 | 0.25 | 1.1 | 4.4 | |
| Chlormethine-N-oxide | 208.6 | 100 | 130 mins | 1.5 | 5 | 60 | 12 | |

* NBP test  † pH 7.5; 37°C  ‡ Yoshida sarcoma  § Monohydrate

One cytotoxic unit (cu) is that amount of a cytotoxic compound which produces survival in 50% of animals after inoculation with tumor cells.

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One cytotoxic unit (cu) is that amount of a cytotoxic compound which produces survival in 50% of animals after inoculation with tumor cells.

enzymes in the liver has been shown by Hill et al, (1972) and Sladek (1973). In addition, Cox et al, (1976a) compared the effect of tissue soluble enzyme fractions on biochemically prepared aldophosphamide, aldophosphamide analogs and phosphorodiamidic acid and showed (a) a range of deactivation abilities with aldophosphamide (liver >> kidney >> intestinal mucosa > tumor > spleen = bovine serum albumin solution); (b) the formation of different amounts of carboxyphosphamide from aldophosphamide; (c) only small reductions in toxicity of phosphorodiamidic acid and 4-hydroxycyclophosphamide by liver cytosol. Correlations were found between the $NAD^+$-dependent aldehyde dehydrogenase activity and the deactivation ability of tissue soluble enzyme fractions. Inhibition of secondary oxidation of aldophosphamide mediated by aldehyde dehydrogenase resulted in diminished deactivation ability *in vitro* and reduced selectivity *in vivo*. These results strongly suggest that enzymic oxidation of aldophosphamide to carboxyphosphamide contributes to the selective action of cyclophosphamide.

b) Reaction of the aldehyde group of aldophosphamide or the hydroxy group of 4-hydroxycyclophosphamide with thiols, as a means of detoxication since the resulting products have only weak alkylating activity and have little toxicity (Hohorst et al, 1976).

Another mechanism of inactivation is the very fast reaction of 4-hydroxycyclophosphamide with thiols to yield 4(S-R)-mercaptocyclophosphamide derivatives. Draeger et al, (1976) showed the reaction of 4-hydroxycyclophosphamide with thiols lead to an equilibrium between the reaction product and the starting materials. Consequently increasing

the thiol concentration would increase the amount of the less toxic 4-hydroxycyclophosphamide derivatives: In addition, Voelcker et al, (1976) showed that considerable amounts of radioactivity are bound to serum proteins after administration of radioactive cyclophosphamide and that 4-hydroxycyclophosphamide was bound to thiol groups of bovine serum albumin yielding a stable product which could be isolated by column chromatography on Sephadex. The product of this thiol reaction can be reactivated to a more toxic form. (Voelcker et al, 1976 and Draeger et al, 1973). Hohorst et al, (1976) suggested that reaction with thiols may lead to a binding of 4-hydroxycyclophosphamide to more sensitive (enzyme) sites in the cells, hence increased cytotoxicity should result, leading to increased specificity.

Thus, although it is likely that phosphorodiamidic acid is the active product, the selective action of cyclophosphamide has not yet been fully explained.

## 1.4. Cellular effects of cyclophosphamide

### 1.4.1. Effect of cyclophosphamide and its metabolite in intact cells:

a) Uptake of cyclophosphamide: Uptake of cyclophosphamide was studied by Goldenberg et al, (1974) who showed that between $0.25 \times 10^{-3}$M and $1.0 \times 10^{-3}$M transport of cyclophosphamide was mediated by a facilitated process. The transport was not inhibited by other alkylating agents, nor by the activated drug or isophosphamide, the close structural analog of cyclophosphamide. One can assume that an activated metabolite of an inactive anticancer agent would be taken up by tumor cells at different

rates. The permeation rate of an alkylating agent should be greater than the alkylation reaction and at the same time the efflux of the substance should be slow enough that it can react with target molecules of the cells. Draeger and Hohorst (1976) studied the uptake of cyclophosphamide metabolites by Ehrlich ascites tumor cells, murine L1210 leukemia cells and mouse L929 fibroblasts and showed that cyclophosphamide and 4-hydroxycyclophosphamide reached nearly the same saturation value, which was significantly lower than nor-nitrogen mustard in all the cells. Their results also indicated that cyclophosphamide could be washed out completely, whereas 4-hydroxycyclophosphamide showed an apparent retention in the cell at 37°C. No investigator has studied the uptake and permeation of phosphorodiamidic acid, because radioactive labelled phosphorodiamidic acid is not yet available. The above findings suggest that there are some differences in permeation rate of alkylating compound as well as selective retention of the molecules within the cells.

b) <u>Cytotoxicity of cyclophosphamide</u>: Recent evidence indicates that the cytotoxicity of cyclophosphamide is due to its active metabolite, phosphorodiamidic acid. Connors et al, (1974b) showed that only phosphorodiamidic acid possessed cytotoxic activity with a biological half-life similar to that of the active antitumor metabolite from microsomal activated cyclophosphamide. In a study of a series of phosphorodiamidic acid derivatives of the general structure shown, Friedman (1967) found

$$\begin{matrix} Cl\text{-}CH_2CH_2 \\ Cl\text{-}CH_2\text{-}CH_2 \end{matrix} \searrow\!\!\nearrow N\text{-}\overset{O}{\overset{\|}{P}} \begin{matrix} \nearrow N \begin{matrix} R_2 \\ R_3 \end{matrix} \\ \searrow O\text{-}R_1 \end{matrix}$$

that esterification of the oxygen ($R_1$) decreased the alkylating activity and cytotoxicity of the molecule, whereas N-substitution ($R_2$ or $R_3$) did not decrease the alkylating activity and cytotoxic activity. Colvin et al, (1976) suggested that the unesterified hydroxyl groups of phosphorodiamidic acid plays a role in alkylation. At physiological pH the OH group bears a negative charge which could be distributed over the molecule, enhancing the basicity of the phosphamide linkage, and the tertiary nitrogen would be nucleophilic enough to allow for formation of an aziridinium ion and subsequent alkylation. On this basis, cyclophosphamide and 4-hydroxycyclophosphamide would be expected to be poor alkylating agents.

c) Cell cycle specificity: The effect of alkylating agents on cells in cycle is not phase specific, synchronized cells in late $G_1$ and S phase seem particularly sensitive to the effects of alkylating agents, leading to their arrest in the $G_2$ phase of the cycle (Casperson et al, 1965; Layde and Baserga , 1964; Levis, 1965; Wheeler et al, 1970). The cells accumulating behind the block in $G_2$ phase have a double complement of DNA while continuing to synthesize other cellular components, such as protein and RNA. This results in unbalanced growth, with the formation of enlarged cells that continue to synthesize DNA (Wheeler et al, 1970) but are not able to divide.

1.4.2. Effects of cyclophosphamide and its metabolite on DNA: The studies of Kohn et al, (1965), Lawley (1966) and Walker (1971) showed that the cytotoxicity of the bifunctional alkylating agents was due to their

reaction with DNA, producing breaks in the DNA molecule and cross-linking of the double stranded DNA, thus interfering with DNA replication and transcription of RNA. The base which is alkylated is guanine, in which N-7 is strongly nucleophilic (Ludlum, 1973; Wheeler, 1973). In addition, less extensive alkylation may occur on the N-1 and N-3 of adenine, the N-3 of cytosine, or the oxygen of guanine. The possible consequences of the reaction of alkylating agents with guanine residues in DNA chains are illustrated in Fig.2 (Calabresi and Parks, 1976). Cross-links between the strands of a DNA double helix hold the two strands together. When DNA is denatured, cross-linked molecules will rapidly renature if returned to favorable condition, while DNA which is not cross-linked does not readily renature, so that these properties can be used to detect such cross-linking (Kohn et al, 1965).

## 1.5. Problems and experimental approaches

4-hydroxycyclophosphamide is more specific in its cytotoxic effects *in vitro* and has a higher therapeutic index *in vivo* but lower alkylating activity as compared to phosphorodiamidic acid. Phosphorodiamidic acid has been identified *in vivo* but not 4-hydroxycyclophosphamide. Since 4-hydroxycyclophosphamide decomposes spontaneously to phosphorodiamidic acid, it is difficult to determine whether one of these compounds or both is responsible for the specific cytotoxic and alkylating activity of the parent compound.

The first objective of this investigation was to measure quantitatively phosphorodiamidic acid generated from microsomal incubation

Figure 2: Mechanism of action of alkylating agents from Calabresi and Parks (1976).

O O — $CH_2$

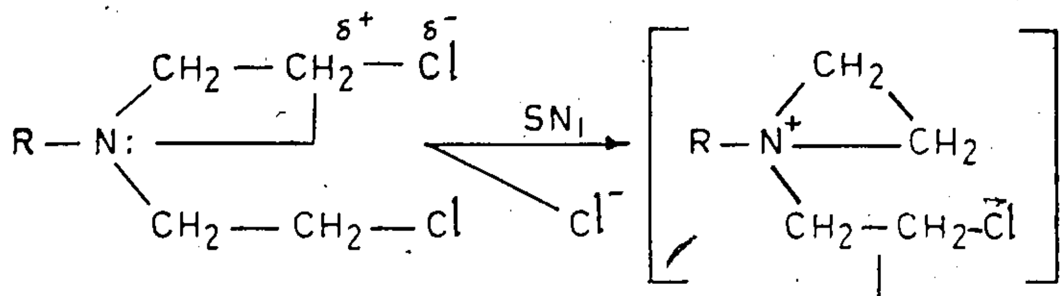
R = — P $CH_2$ = CYCLOPHOSPHAMIDE

N — $CH_2$

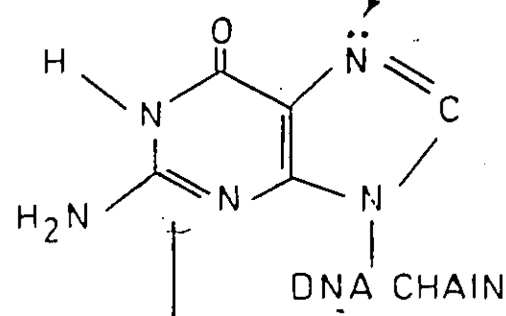
O

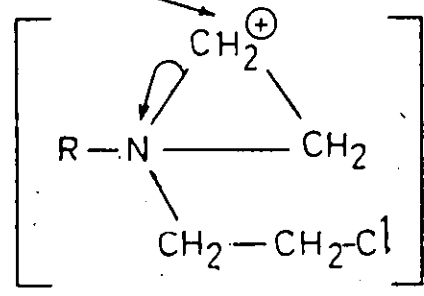
R = — P — $O^{\ominus}$ = PHOSPHORODIAMIDIC ACID

$NH_2$

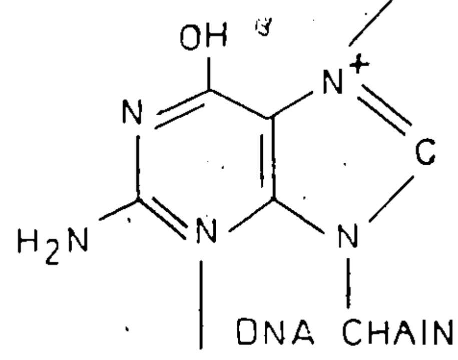


KETOTAUTOMER
OF. GUANINE

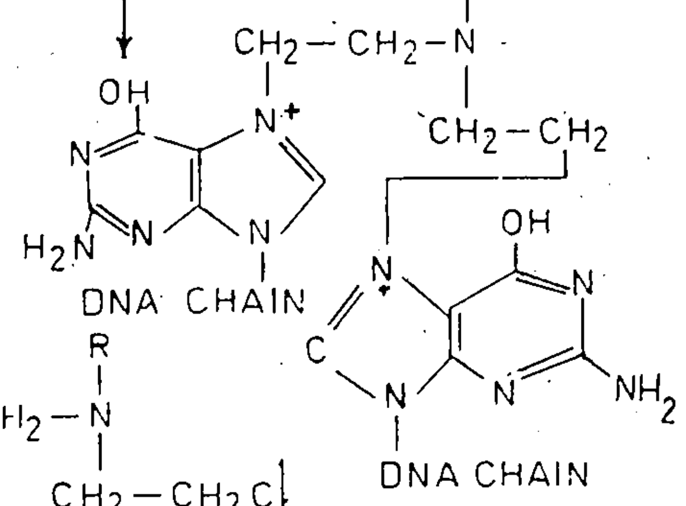


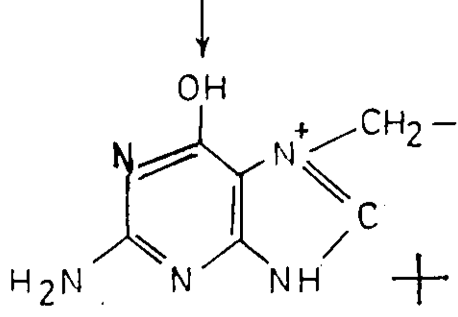
ENOLTAUTOMER




CROSS LINKING WITH
2 ND GUANINE




DEPURINATION



DEPURINATED DNA CHAIN

of cyclophosphamide and determine the effects of the incubation product on tissue culture in comparison with the effect of pure phosphorodiamidic acid.

Both cyclophosphamide and phosphorodiamidic acid have the β-chloroethyl side chain available for alkylation. The second objective of this investigation then was to compare the degree of cross-linking of DNA in intact cells and nuclei when incubated with cyclophosphamide and phosphorodiamidic acid.

# 2. REAGENTS AND METHODS

## 2.1. Reagents and materials

Cell culture materials were obtained from Grand Island Biological Company (Grand Island, NY) and the following compounds from the sources given: nicotinamide adenine dinucleotide phosphate (oxidized form), glucose-6-phosphate dehydrogenase, ribonuclease, protease, mitomycin-c, calf thymus DNA, RNA, 8-hydroxyquinoline, dextran 500, Sigma Chemical Company, (St. Louis, MO); 4-p-nitrobenzyl-pyridine, m-aminophenol, Aldrich Chemical Company (Milwaukee, WI); acrolein, Eastman Kodak Company (Rochester, NY); cyclophosphamide monohydrate (pure) Koch Light Laboratories Limited (Colnbrook, Bucks., England); phosphorodiamidic acid (NSC No. 69945), N,N, bis(2-chloroethyl) with cyclohexylamine, National Cancer Institutes (Bethesda, MD); hydroxylamine hydrochloride, Fisher Scientific Company (Fairlawn, NJ); Darvan 1, R.T. Vanderbilt Company Inc., (Norwalk, CT); [methyl-$^{3}$H]-thymidine, specific activity 5 curies/mmoles, naphthalene, Amersham/Searle (Arlington Heights, IL); sodium phenolbarbital injection, Winthorp Laboratories (Aurora, Ont., Canada); bovine serum albumin, Calbiochem. (San Diego, CA); dioxane scintillation grade, Mallinckrodt (St. Louis, MO); perchlcric acid, BDH Chemicals (Toronto, Ont.) and ethoxy ethanol, Matheson, Coleman and Bell (Norwood, OH); carbowax 6000, Analabs (North Haven, CT).

## 2.2. Cell source

Cell lines LS2 and LM4 are the sublines of L1210 mouse leukemic cells described by Hillcoat (1971), sensitive and resistant respectively to the drug Methotrexate. The cells are maintained in suspension culture in RPMI 1640 medium containing 10% fetal calf serum by subculturing $2 \times 10^5$ cells per ml in 25 $cm^2$ (50 ml) Falcon tissue culture flasks. All cell concentrations were determined using a Model F coulter counter. The growth curves of LS2 and LM4 cells were determined by counting the number of cells every 24 hours over a period of 6 days.

## 2.3. Determination of the effect of cyclophosphamide and phosphorodiamidic acid on intact cells

### 2.3.1. Dose dependent effect of cyclophosphamide and phorphorodiamidic acid:

Cells from LS2 and LM4 lines were counted, centrifuged at 600 x g for 5 minutes, then resuspended at $2 \times 10^5$ cells per ml in medium containing 10% fetal calf serum and different concentration of drugs (cyclophosphamide or phorphorodiamidic acid) ranging from $10^{-3}$M to $10^{-7}$M. The drugs were dissolved in medium immediately before use and were sterilized by filtration through a Millipore (0.22 μm) filter. The cells were continuously exposed to the drugs during growth at 37°C and were counted on the fourth day.

### 2.3.2. Effect of the drug at various stages of cell growth:

Three day old LS2 and LM4 cells were resuspended in medium containing 10% fetal calf serum and 0.1 ml of $10^{-2}$M phosphorodiamidic acid or 0.1 ml or $10^{-1}$M

of cyclophosphamide was added to 9.9 ml of suspension cultures at 0, 24, 48 and 72 hours. Controls were cells grown without the drugs. The cells were counted in each case on the fourth day.

2.3.3. Effect of the drugs after various times of exposure: LM4 and LS2 cells were allowed to grow for 24 hours after subculturing. Phosphorodiamidic acid and cyclophosphamide were added to the cultures to give final concentrations of $10^{-4}$M and $10^{-3}$M respectively. After 0, 1, 2, 3, and 4 hours incubation of the cells with the drugs, the cells were spun down at 600 x g for 5 minutes, washed once with 5 ml of medium, again spun down at 600 x g for 5 minutes, then resuspended in 10 ml of medium containing 10% fetal calf serum. Controls without the drugs were treated similarly and all cells counted on the fourth day.

## 2.4. Determination of cell viability

2.4.1. Trypan blue dye exclusion test: The first test was based on the ability of living cells to exclude the dye, trypan blue. The method of Phillips (1973) was followed. One ml of the cell suspensions were spun down, washed once with medium and resuspended in 1 ml Hank's solution. Then 0.1 ml of 0.4% trypan blue solution was added and mixed 10 times with a Pasteur pipette. After 4 minutes at room temperature, a drop of the suspension was placed in a cell counting chamber (C.H. Hausser and Son). The number of stained cells and non-stained cells in a given area was determined and the number of viable, non-stained cells was expressed as a percentage of the total number of cells counted. For each deter-

mination, the total number of cells counted was 100.

2.4.2. Colony forming assay: The plating mixture in 25 $cm^2$ Falcon culture flasks contained 40% of 2.2% sterile methyl cellulose in medium, 10% fetal calf serum, 40% cell suspension to give appropriate final cell concentration between 20 to 4000 cells per ml, 8% of conditioned medium and 2% killed cell suspension of 1 x $10^6$ cells per ml.

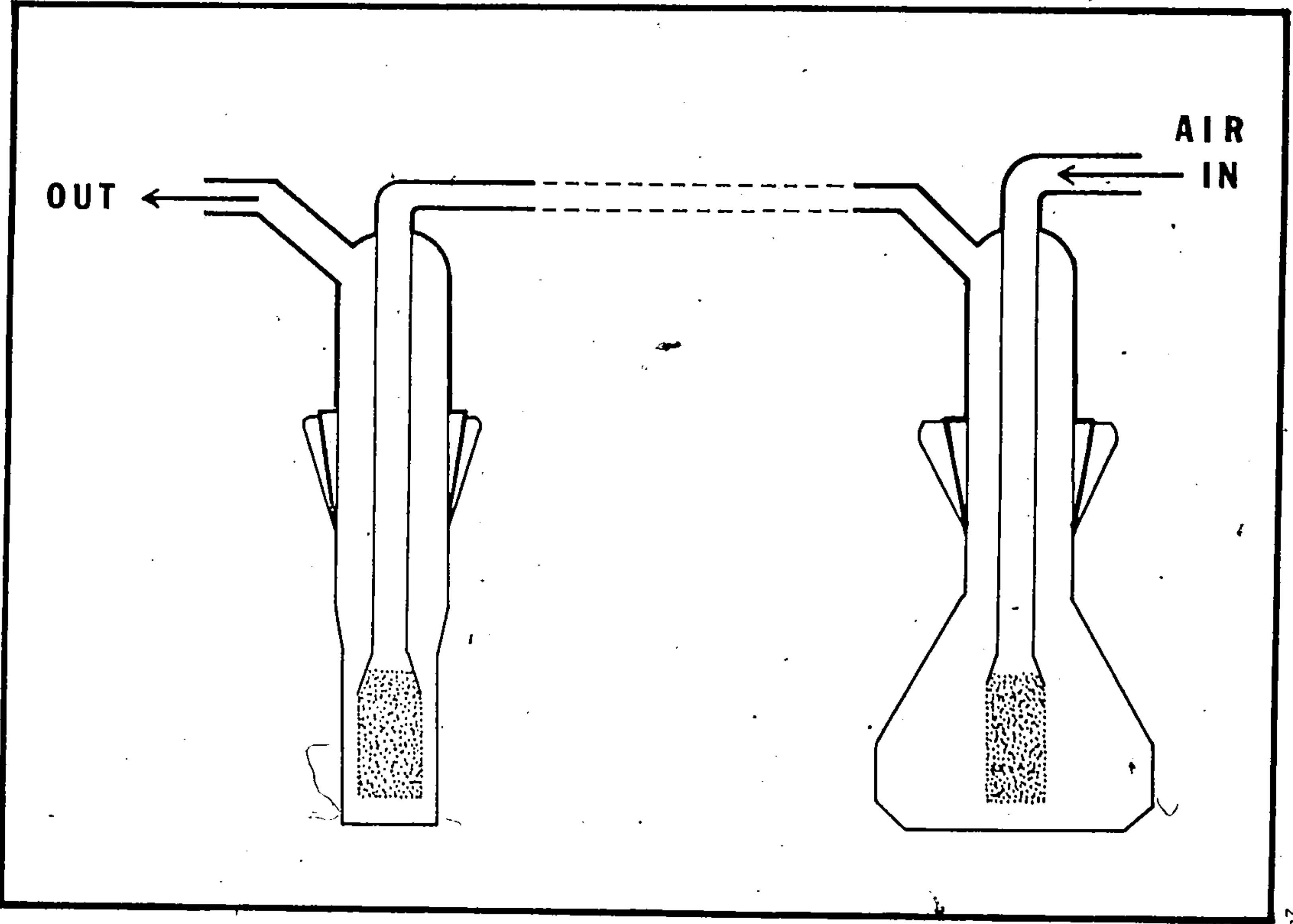
Killed cells were used to promote the formation of colonies (Macpherson and Bryder, 1971). LM4 cells were grown for 24 hours and a solution of mitomycin in medium was added to give a final concentration of 2 μg per ml. The cells were grown for an additional 24 hours, spun down at 600 x g for 10 minutes, washed once with medium and resuspended in fresh medium to give a final concentration of 1 x $10^6$ cells per ml.

## 2.5. Metabolism of cyclophosphamide

Male Wistar rats, weighing 200 - 250 g, were used for studies of metabolism of cyclophosphamide. Microsomal fractions were prepared from rats livers as described by Connor et al, (1974b). Sodium phenobarbital was administered in their drinking water at a level of 500 mg per litre for at least 10 days prior to each experiment to enhance mixed function oxidase activity in the liver. The rats were decapitated and the livers were removed immediately and chilled in ice. Rat liver was homogenized in four volumes of cold 0.15 M KCl using a tissue press, then the homogenate centrifuged at 12,000 x g for

35 minutes. The supernatant was collected and centrifuged at 190,000 x g for 35 minutes at $0^{o}C$. The microsomal pellet was resuspended in 0.15 M cold KCl and recentrifuged at 190,000 x g for 35 minutes at $0^{o}C$. The pellet was then suspended in 0.2 M Tris-HCl buffer, pH 7.4, such that 1 ml of suspension was equivalent to 5 g of original liver. The microsomes could be stored at this point at $-20^{o}C$, if necessary. Incubations were carried out at $37^{o}C$ for 45 minutes in 50 ml Erlenmeyer flasks, so that a stream of air was directed through the reaction mixture. The reaction flask was connected with teflon tubing (3 mm inner diameter) to a trapping flask containing reagent which reacted with acrolein. Figure 3 shows the reaction and trapping flasks. The reaction flask contained 3 ml of microsomal suspension, 0.52 mM of nicotinamide adenine dinucleotide phosphate ($NADP^+$), 15 mM of glucose-6-phosphate, 4 mM of $MgCl_2 \cdot 6H_2O$, 90 units of glucose-6-phosphate dehydrogenase and 18 mM of cyclophosphamide in a total volume of 30 ml buffered at pH 7.4 with 0.1 M Tris-HCl. Glucose-6-phosphate dehydrogenase catalyzes the reaction glucose-6-phosphate + $NADP^+ \rightleftharpoons$ 6 phosphogluconate + NADPH + $H^+$ The net result being the production of NADPH for reduction of cyclophosphamide to 4- hydroxy cyclophosphamide. The trapping flask, which was chilled in an ice bath during the incubation contained 3 ml freshly prepared reagent (250 mg of m-aminophenol plus 300 mg of hydroxylamine hydrochloride dissolved in 25 ml of 1 N HCl). At the end of the incubation, the reaction mixture was placed on ice then centrifuged at 100,000 x g for 30 minutes to remove the microsomes. The supernatant was kept for further analysis. The trapped acrolein was subjected to fluorometric analysis

Figure 3: The reaction and trapping flasks used in microsomal incubation of cyclophosphamide.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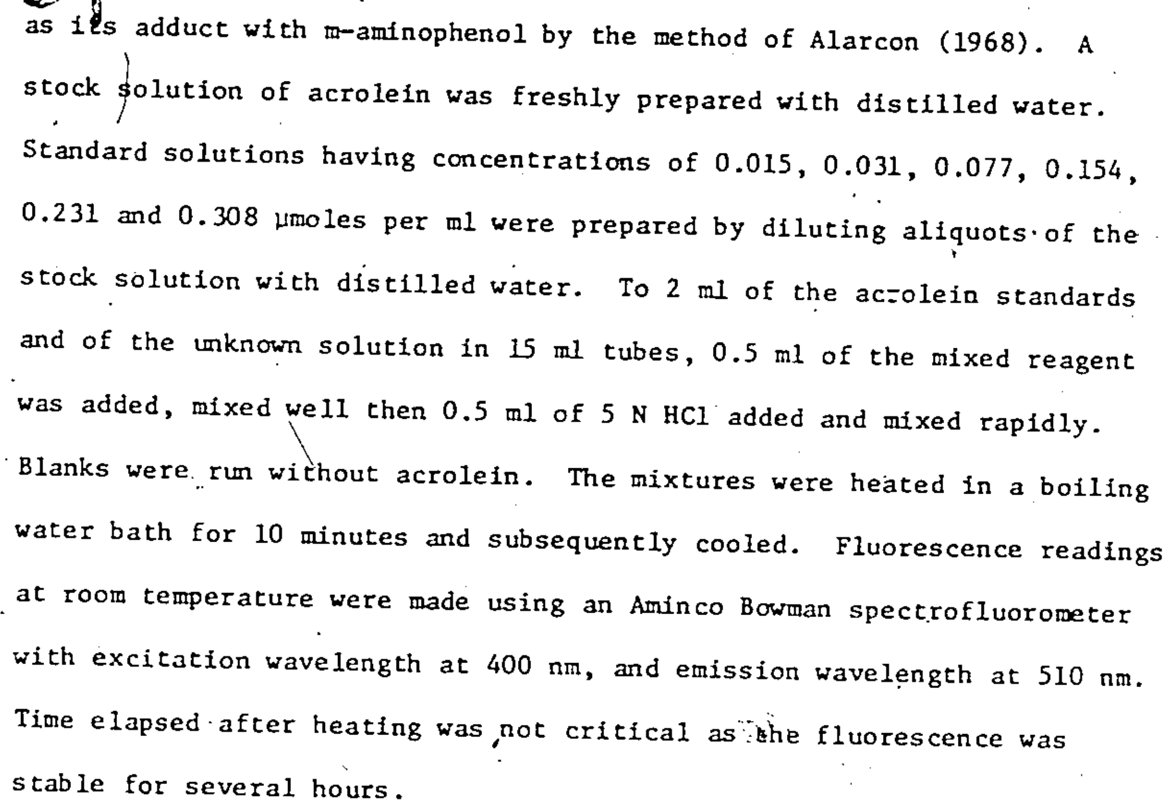


as its adduct with m-aminophenol by the method of Alarcon (1968). A stock solution of acrolein was freshly prepared with distilled water. Standard solutions having concentrations of 0.015, 0.031, 0.077, 0.154, 0.231 and 0.308 µmoles per ml were prepared by diluting aliquots of the stock solution with distilled water. To 2 ml of the acrolein standards and of the unknown solution in 15 ml tubes, 0.5 ml of the mixed reagent was added, mixed well then 0.5 ml of 5 N HCl added and mixed rapidly. Blanks were run without acrolein. The mixtures were heated in a boiling water bath for 10 minutes and subsequently cooled. Fluorescence readings at room temperature were made using an Aminco Bowman spectrofluorometer with excitation wavelength at 400 nm, and emission wavelength at 510 nm. Time elapsed after heating was not critical as the fluorescence was stable for several hours.

## 2.6. Isolation and identification of metabolites

After removal of the microsomes from the reaction mixture, protein was precipitated by addition of 4 volumes of 100% ethanol and removed by centrifugation at 1200 x g. The supernatant was evaporated to almost dryness in a rotary evaporator at 30°C, connected to water suction. The volume of the concentrated supernatant was adjusted to 4 ml with distilled water and the pH of the concentrate was adjusted to pH 4.0 by addition of 0.1 M HCl and the solution was extracted with 5 ml chloroform. The mixture was shaken for 20 minutes using a wrist action shaker (Burrell model 75), then centrifuged at 1000 x g for 10 minutes. The chloroform layer was collected. This was repeated twice then all the chloroform

extracts combined and evaporated to dryness at room temperature under nitrogen. Then 0.5 ml of methanol was added to the extract which was subjected to thin layer chromatography on 20 x 10 cm plates of 0.25 mm (precoated with silica gel G-25) for identification purpose. Plates 20 x 20 cm were used for preparative purposes. The solvent was chloroform-ethanol (9:1). The plates were dried at room temperature and the 20 x 10 cm plates sprayed with 1% of 4-p-nitrobenzylpyridine (NBP) in acetone, heated in an oven for 20 minutes at 120°C then sprayed with 3% KOH in methanol. The alkylating agent appeared as blue spots. Silica gel on the non-sprayed plate was removed from regions corresponding in Rf values to those of the detected substances and eluted with three lots of methanol. This pooled eluate was evaporated to almost dryness under nitrogen at room temperature and the volume adjusted to 1 ml with MeOH. An aliquot of 100 µl was treated with excess diazomethane until the solution stayed yellow. The reacted solution was taken to dryness under nitrogen, diluted in 100 µl MeOH then an aliquot of 20 µl was spotted on thin layer plate. Gas chromatographic analyses were performed on a Varian 2100 gas chromatograph with a six foot column containing 1.5% OV 17 on Chromosorb W High Performance (HP), 80/100 mesh with temperature at 220°C. Injector port and detector were kept at 300°C with a setting of 40 on the flow gauge (flow gauge adjusted to give ml/min flow rate for helium). Gas chromatography/mass spectrometry (GC-MS) data were obtained on a Varian CH-7 Spectrometer coupled to a Varian 2700 gas chromatograph by a Watson-Biemann separator. The conditions of the gas chromatograph in the GC-MS were kept the same as the conditions of

the regular GC, and the flow rate adjusted to give maximum vacuum of $10^{-6}$ torr. Mass spectral conditions were set at an ionizing voltage of 70 eV, emission current of 300 µA and ion source temperature at 290°C. Mass spectra were recorded on a Varian 620L computer. The rest of the sample was evaporated to dryness under nitrogen and resuspended in 1 ml of distilled water, to be used for Epstein's colorimetric determination.

2.6.1. <u>Estimation of the total amount of phosphorodiamidic acid produced</u>: Quantitative determination of phosphorodiamidic acid extracted from the liver microsomal incubation was determined by Epstein's colorimetric determination as described by Friedman et al. (1961). Phosphorodiamidic acid stock solution was prepared by weighing 1 mg and diluting to 10 ml with distilled water. Standard solutions having concentrations of 1, 2.5, 5, 10, 25, 50 and 100 µg per ml were prepared by diluting aliquots of the stock solution with distilled water. One ml aliquots of the standard and sample were used for determination. All tubes were chilled in ice, and 1 ml of 0.1 M acetate buffer, pH 4.6, was added and mixed well. The solution was treated with 0.4 ml of 5% (w/v) NBP reagent in acetone, mixed well and heated in boiling water for 20 minutes, then cooled in ice. Two ml acetone was added to the mixture followed by 5 ml ethyl acetate and mixed well. Each tube was wrapped in foil to eliminate as much light as possible then 1.5 ml of 0.25 N NaOH was added, the mixture was shaken vigorously by hand about 30 times and let stand for 1 minute. The top layer was taken for absorbance measurement at 540 mµ on a Turner colorimeter. Because of the instability of the colour formed,

the operation from the introduction of NaOH to the reading in the colorimeter was carried out rapidly and in a darkened room using only indirect light from a 40 watt incandescent bulb. A blank was run without phosphorodiamidic acid. To improve the sensitivity and specificity of quantitation of phosphorodiamidic acid an extractive alkylation method was also attempted (Ervik and Gustavii, 1971), to produce a derivative suitable for gas chromatographic and mass spectral analysis.

2.6.2. Determination of the stability of cyclophosphamide and phosphorodiamidic acid in incubation condition: Stability of cyclophosphamide and phosphorodiamidic acid in incubation medium during 4 hours of incubation was determined by adding 0.2 ml of $10^{-1}$M cyclophosphamide or $10^{-2}$M phosphorodiamidic acid to 19.8 ml of medium containing 10% fetal calf serum and 4 x $10^{6}$ LM4 cells had been grown for 24 hours in suspension. Two ml aliquots were withdrawn from the suspension at 0, 30, 60, 90, 120, 150, 180, 210 and 240 minutes. The incubation was carried out at 37°C, then the tubes were centrifuged at 600 x g for 5 minutes and the supernatant was collected. Cyclophosphamide samples of 20 µl were spotted on thin layer plate. Four volumes of 100% EtOH were added to the phosphorodiamidic acid samples, the mixture was shaken vigorously then centrifuged at 1000 x g for 5 minutes. The supernatant was collected and evaporated to dryness in a rotary evaporator at 30°C under water suction. The extract was dissolved in 1 ml of distilled water and adjusted to pH 4.0 using 0.1 N HCl. The acidified aqueous solution was extracted with 3 ml chloroform, shaken for 20 minutes, centrifuged at 1000 x g for 5 minutes, then the

chloroform layer collected. This procedure was repeated twice, all the chloroform layers were combined and evaporated to dryness at room temperature under nitrogen. The extract was dissolved in 200 µl of MeOH, reacted with diazomethane and the reacted solution was used for spotting on thin layer plates.

2.7. Intranuclear reactivity of cyclphosphamide and phosphorodiamidic acid

2.7.1. Prelabelled cells: LM4 cells, at 2-2.5 x $10^5$/ml, were grown for 24 hours under the usual conditions but with 5 µM unlabelled thymidine plus 0.1 µCi/ml [methyl-$^3$H] thymidine. When larger quantities of unlabelled cells were required, they were grown in $BDF_1$ mice by intraperitoneal injection of 4 x $10^6$ cells per mouse. Five days after innoculation the mice were sacrificed by ether inhalation and the cells from peritoneal cavity washed with saline solution, centrifuged at 600 x g for 10 minutes and the pellet was collected.

2.7.2. DNA alkylated in intact cells treated with cyclophosphamide and phosphorodiamidic acid: The cells were grown in flasks for 24 hours with tritiated thymidine to label DNA. Average total amounts of DNA per flask was 39.8 $\pm$ 0.7 µg. At that point cyclophosphamide or phosphorodiamidic acid was added to give final concentrations of $10^{-3}$ or $10^{-4}$M respectively. After four hours incubation at 37°C, the suspension cultures were centrifuged at 600 x g for 10 minutes and washed once with medium. Control cultures were prepared by adding medium to the culture in place of the drug solution. The cells could be stored in the freezer if necessary.

2.7.3. DNA alkylated in nuclei treated with cyclophosphamide and phosphorodiamidic acid: Nuclei from LM4 cells were prepared using the method of Rickwood et al. (1973). The prelabelled cells were suspended at approximately 1 x $10^8$ cells per ml in 0.001 M Tris-HCl (pH 7.4) containing 3 mM $MgCl_2$ and 0.05% (w/v) Darvan No. 1 (surface active agent) then homogenized with 15strokes in a Potter Elvehjem homogenizer with a tight fitting teflon pestle. The cell homogenate was diluted with sucrose buffer (1.25 M sucrose, 0.2 M Tris-HCl, pH 7.4, 15 mM $MgCl_2$) to give a final sucrose concentration of 0.25 M. The nuclei were then sedimented at 1000 x g for 10 minutes at $0^oC$ and washed twice in 0.25 M sucrose containing 3 mM $MgCl_2$. The nuclei were finally sedimented through 2.2 M sucrose containing 3 mM $MgCl_2$ and 5 mM Tris-HCl, pH 7.4 by centrifugation at 44,000 x g for 1 hour. The nuclei were suspended in medium to give concentration of 200 μg of DNA/ml. Carrier nuclei were prepared from LM4 cells grown in $BDF_1$ mice.

A suspension of 2 ml of nuclei, 1 ml of fetal calf serum and 6.9 ml of medium in a 25 ml Erlenmeyer flask was treated with 0.1 ml of $10^{-1}$M of cyclophosphamide or 0.1 ml of $10^{-2}$M phosphorodiamidic acid Controls were set up by adding 0.1 ml of medium instead of the drug solution. All the flasks were incubated at $37^oC$ for four hours with constant shaking and at the end of incubation, 5 ml of cold medium and the carrier nuclei were added. The suspension was centrifuged at 1000 x g for 10 minutes at $0^oC$, washed once with medium and the pellet was collected for DNA isolation.

2.7.4. Isolation of DNA: DNA was isolated from intact cells as well as

from the nuclei using the method of Walker (1971). Ten ml of cold 5% (w/v) aqueous 4-amino sodium salicylate was added to 2 x $10^8$ cells or nuclei isolated from 4 x $10^8$ original cells. The cells were dispersed and 1/10 volume of 10% aqueous sodium dodecyl sulfate was added, the mixture was kept in room temperature for approximately 20 minutes to allow for complete lysis of the cells. A solution of ribonuclease at a concentration of 1 mg/ml in 0.1 M NaCl - 0.01 M acetate, pH 5.0 was prepared. Three ml of this solution was added per 100 ml of lysate. The lysate was incubated at 37°C for 20 minutes, then protease (in 0.1 M NaCl - 0.01 M acetate, pH 5.0) was added to give a final concentration of 0.5 mg/ml. The resulting mixture was incubated for two hours at 37°C. Ten ml of phenol reagent (500 g phenol, 55 ml of distilled water and 0.6 g 8-hydroxyquinoline) were added and the mixture was shaken by a wrist action shaker for 20 minutes, then centrifuged at 1250 x g for 20 minutes. The upper phase was carefully removed and an equal amount of phenol reagent was added and the extraction procedures were repeated. The resultant solution was further deproteinized by shaking with an equal volume of $CHCl_3$-isoamyl alcohol (24:1), centrifuging at 1250 x g for 20 minutes, collecting the upper phase and repeating the $CHCl_3$-isoamyl alcohol procedure. The upper phase was dialyzed against three changes of 0.1 M Na-acetate. To the dialyzate, equal amounts of ethoxy ethanol were added and DNA was obtained on a glass rod by stirring. The isolated DNA was washed with 50% aqueous-ethoxy ethanol and dissolved in 0.01 M phosphate buffer, pH 6.8 such that 1 ml of suspension was equivalent to 1.5 x $10^8$ of original cells. If the DNA aqueous-ethoxy ethanol mixture

was too dilute to precipitate the DNA, it was concentrated on a rotary evaporator at 20$^{o}$C with water suction. DNA concentration was estimated by the method of Burton (1956), protein by the method of Lowry (1951) and RNA by the orcinol procedure.

2.7.5. Estimation of inter-strand cross-linking of DNA: Denaturation of DNA results in strand separation except when cross linking between strands is present. On rapid cooling, the separated single strands do not renature. However, those strands held in proximity by cross linking do renature under these conditions, reforming double-stranded DNA. Thus the amount of double-stranded DNA present is a measure of the amount of inter-strand cross-linking present. To measure the amount of double-stranded DNA present, the poly-ethylene glycol system of Alberts (1967) has been used. Double-stranded DNA partitions into the polyethylene glycol rich upper phase and single-stranded DNA partitions in the lower phase. The isolated DNA was diluted in 10 mM phosphate buffer, pH 6.8 to give an approximate concentration of 400-500 μg per ml. The DNA was denatured by carefully mixing with an equal volume of 0.1 N NaOH, incubating at 37$^{o}$C for 10 minutes, cooling and adding an equal volume of ice cold 0.1 M $NaH_2PO_4$. The cold solution was dialyzed at 4$^{o}$C against 10 mM sodium phosphate, pH 6.8 with three changes of the buffer. The DNA samples were sonicated for one minute (3 x 20 second intervals) at 0$^{o}$C, 30 KHz and partitioned in the two phase system of Alberts (1967).

The two phases system was prepared by t[illegible]ng a tared beaker plus a stirring bar and dissolving 18.5 g of dextran 500 in 8.15 ml of distilled hot water near boiling, cooling and adjusting the weight of the flask to 100 g with distilled water. Of this solution, 3.2 g were

removed and replaced with 9.2 g of polyethylene glycol (carbowax 6000). The mixture was stirred until all the carbowax dissolved and the phases were then separated by centrifugation for 20 minutes at 1250 x g. When kept at 4°C, the preparation was stable.

Partition of single and double-stranded DNA was carried out by mixing 3 ml of the DNA solution, 1.20 ml of lower phase and 1.28 ml of upper phase in 15 ml stoppered ice chilled tubes. Because the lower phase was very viscous, it was measured and transferred with a graduated disposable syringe. The mixture was shaken vigorously and left overnight in the cold with occasional stirring on a Vortex mixer. Efficiency of partition was checked by using native double-stranded DNA. The amount of DNA extracted into the upper phase was measured by adding 250 µl of the sample to 10 ml of Bray's scintillation solution and counting the sample in a Beckmann scintillator, while the unfractionated DNA solution was also counted in the same way. The percentage of DNA extracted was calculated from these values. Quench corrections were determined from a calibration curve.

## 3. RESULTS

### 3.1. Effect of cyclophosphamide and phosphorodiamidic acid in intact cells

The growth rates of LM4 and LS2 cells in suspension cultures are shown in Figure 4. The two cell lines have similar growth characteristics. Both cell lines reached resting phase after three days, hence the cells were subcultured on the 3rd day and counted on the 4th day of each subsequent experiment, unless otherwise stated.

Figure 5 and 6 show the inhibition of cell growth as a function of drug concentration, when the drugs were added at the time the cells were subcultured so that the cells were continuously exposed to the drugs. The sensitivity of LM4 or LS2 to cyclophosphamide and phosphorodiamidic acid is the same. Phosphorodiamidic acid concentrations of $1 \times 10^{-4}$M or greater produced 90% inhibition of growth while 50% inhibition was found at $1 \times 10^{-5}$M. Cyclophosphamide at $1 \times 10^{-4}$M showed no effect on LM4 or LS2 cell growth and only 15% growth inhibition in LS2 cells and 8% in LM4 cells (at $1 \times 10^{-3}$M cyclophosphamide). These results suggested that phosphorodiamidic acid is at least 100 times more potent than cyclophosphamide in inhibiting cell growth.

The effect of $1 \times 10^{-4}$M phosphorodiamidic acid and $1 \times 10^{-3}$M cyclophosphamide at various stages of cell growth were investigated in LS2 and LM4 cells. Figures 7 and 8 show that the effect of cyclophosphamide

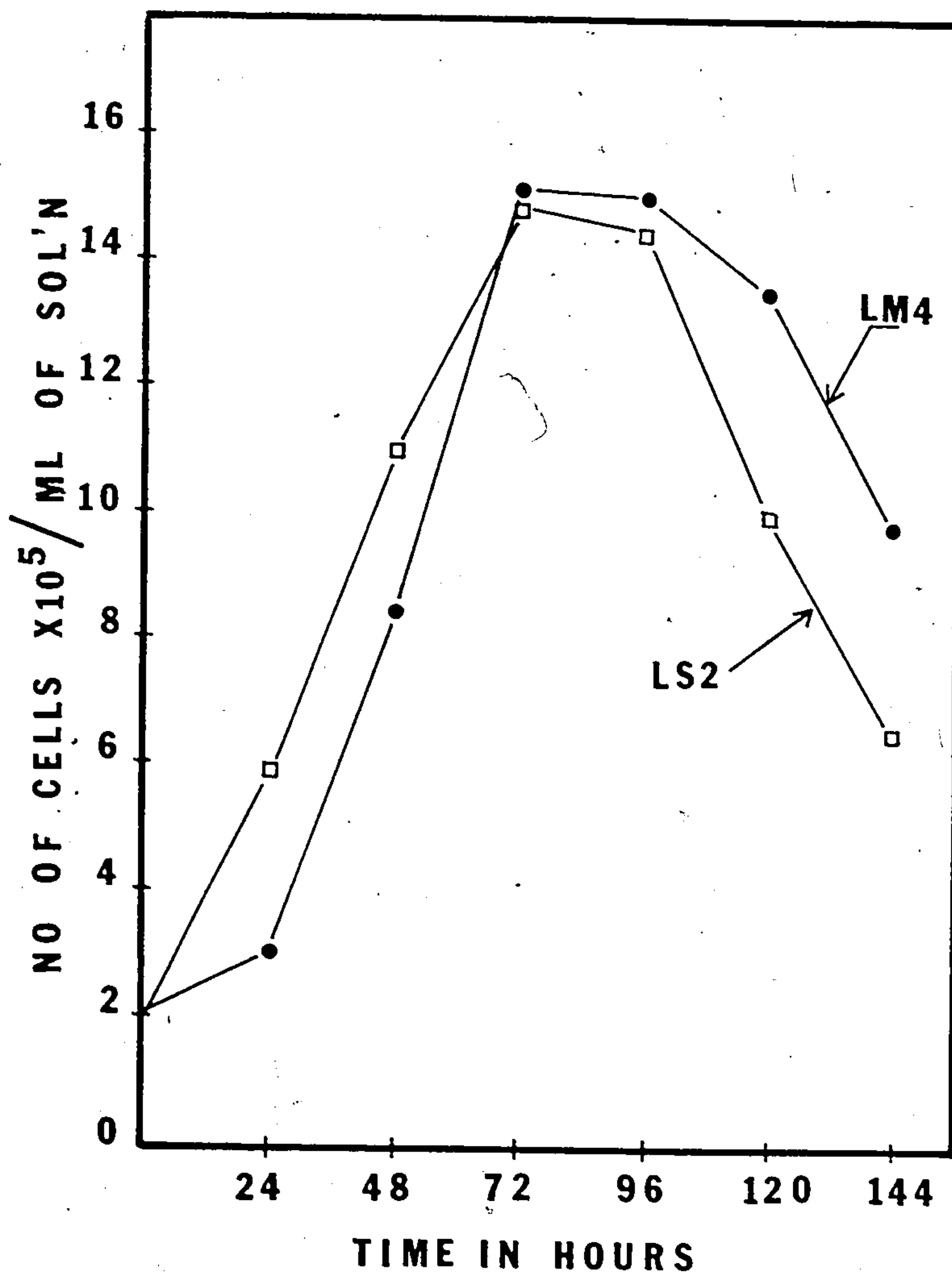



Figure 4: Growth curve of LS2 and LM4 cells
LS2 and LM4 cells, 2 x $10^6$ were grown in 10 ml of 1640 medium containing 10% fetal calf serum. The cells were counted every 24 hours up to 144 hours and the flasks set up in triplicate.

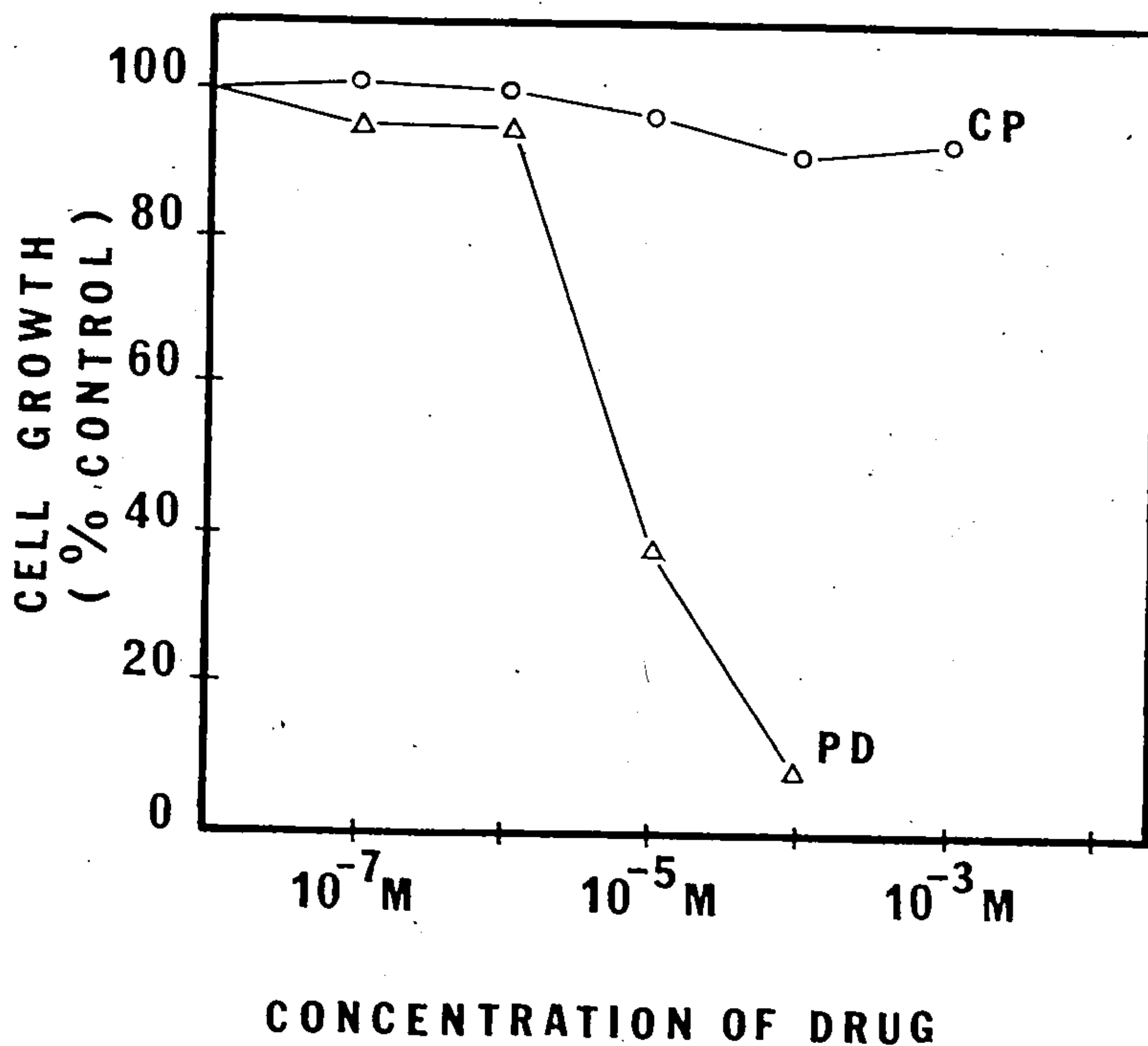



Figure 5: Effect of varying concentration of cyclophosphamide and phosphorodiamidic acid on LM4 cells.
Cells were grown in the presence of varying concentrations of cyclophosphamide or phosphorodiamidic acid, added at the time the cells were subcultured. Cells were allowed to grow until the control cells reached resting phase and were then counted. Each point is the average of three experiments and is expressed as a percentage of the control cells.

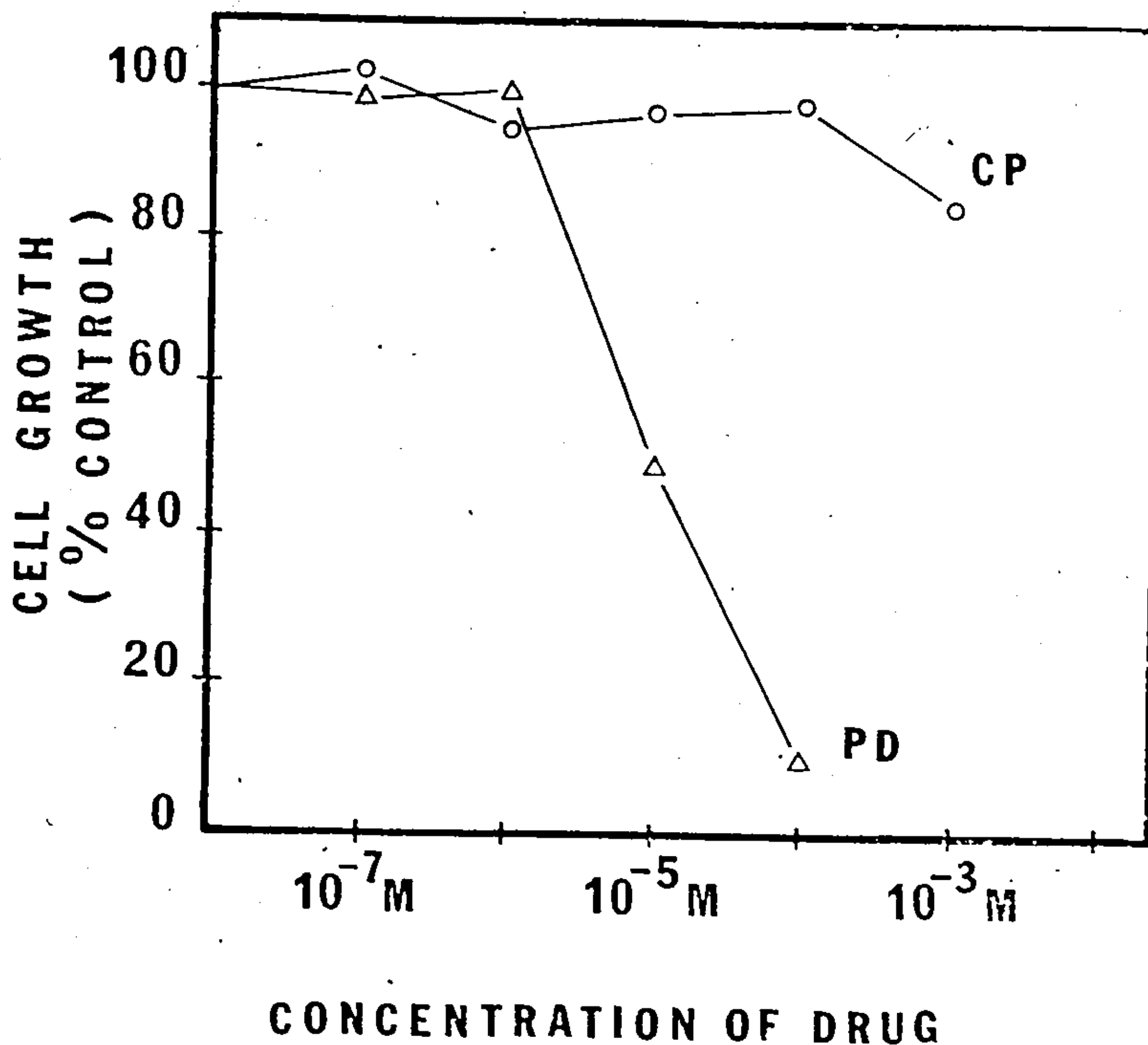



Figure 6: Effect of varying concentrations of cyclophosphamide and phosphorodiamidic acid on LS2 cells.
Cells were grown in the presence of varying concentrations of cyclophosphamide or phosphorodiamidic acid, added at the time the cells were subcultured. Cells were allowed to grow until the control cells reached resting phase and were then counted. Each point is the average of three experiments and is expressed as a percentage of the control cells.

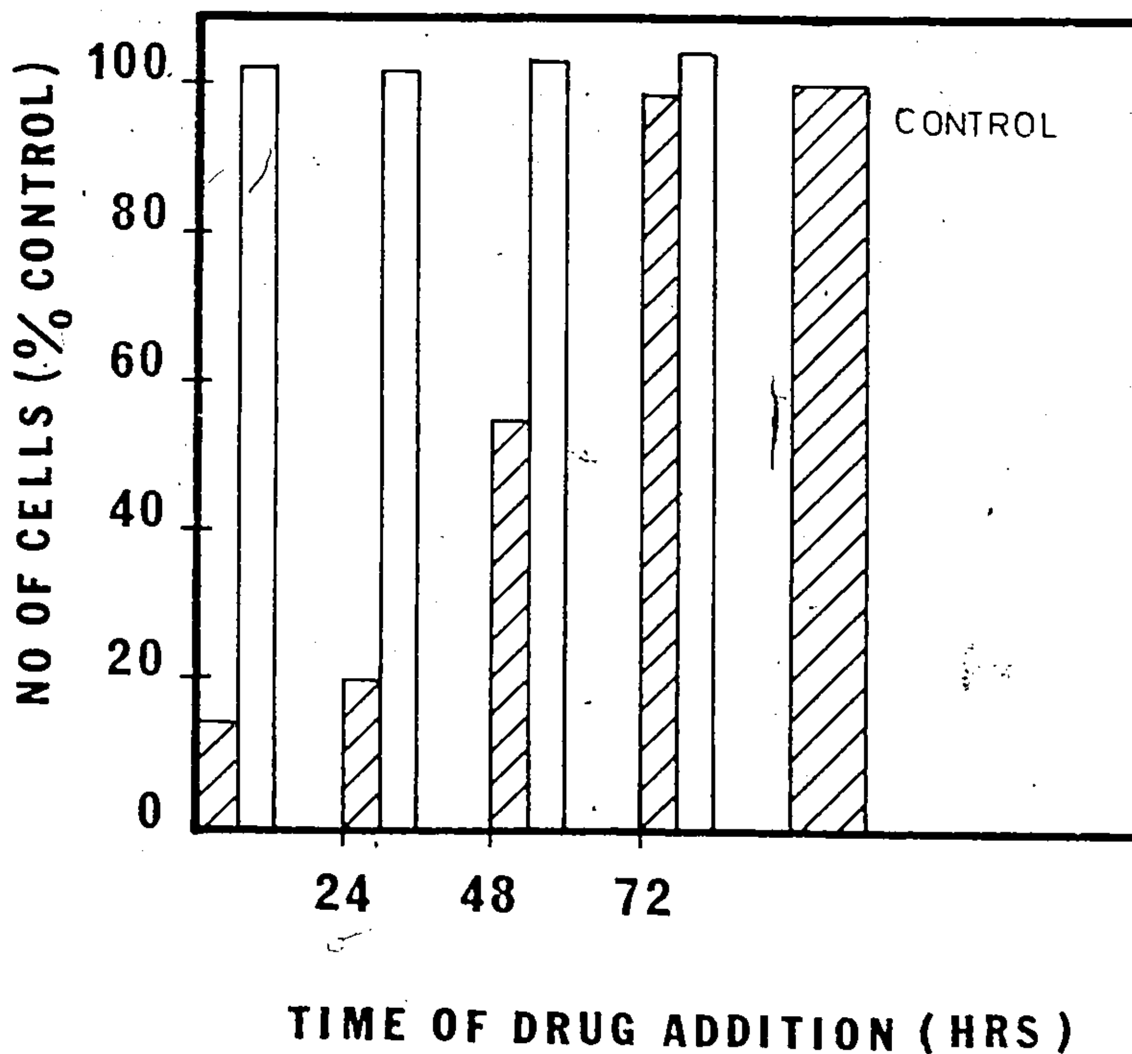



Figure 7: Effect of cyclophosphamide at various stages of LM4 cell growth.
0.1 ml of $10^{-1}$M cyclophosphamide was added to 9.9 ml of LM4 cells at 0, 24, 48 and 72 hours from the time the cells were subcultured. Control cells were grown without the drug. The total number of cells were counted before the drug was added (▨), at 0, 24, 48 and 72 hours. The cells were allowed to grow until the control cells reached the resting phase (4 days). Total number of cells were counted (▭) and the results were expressed as the percentage of the control cells. Each point represents the average of three experiments.

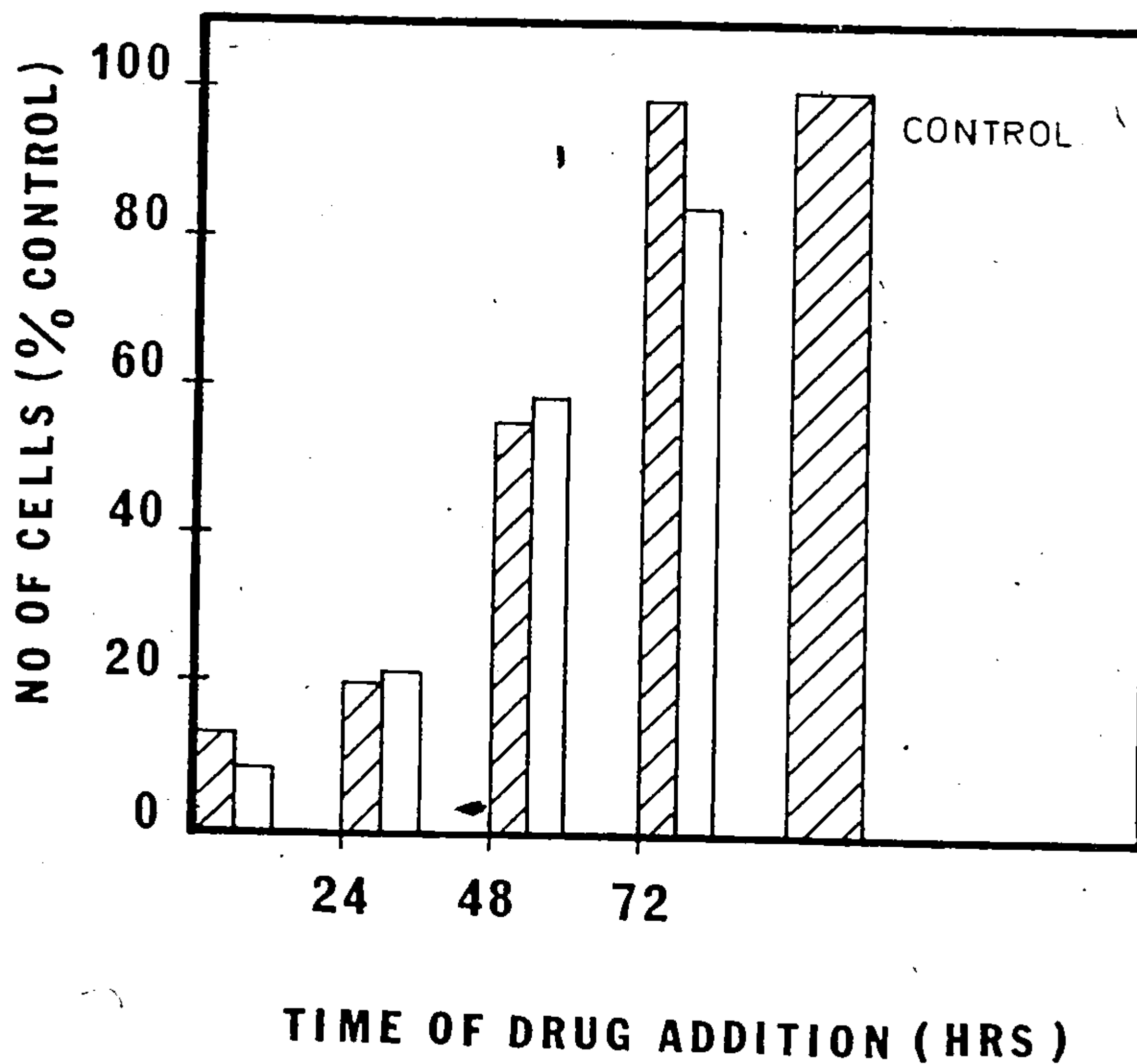



Figure 8: Effect of phosphcrodiamidic acid at various stages of LM4 cell growth.
0.1 ml of $10^{-2}$ of phosphorodiamidine was added to 9.9 ml of LM4 cells at 0, 24, 48 and 72 hours from the time the cells were subcultured. Control cells were grown without the drug. The total number of cells were counted before the drug was added (▨), at 0, 24, 48 and 72 hours. The cells were allowed to grow until the control cells reached the resting phase (4 days). Total number of cells were counted (▭) and the results were expressed as the percentage of the control cells. Each point represents the average of three experiments.

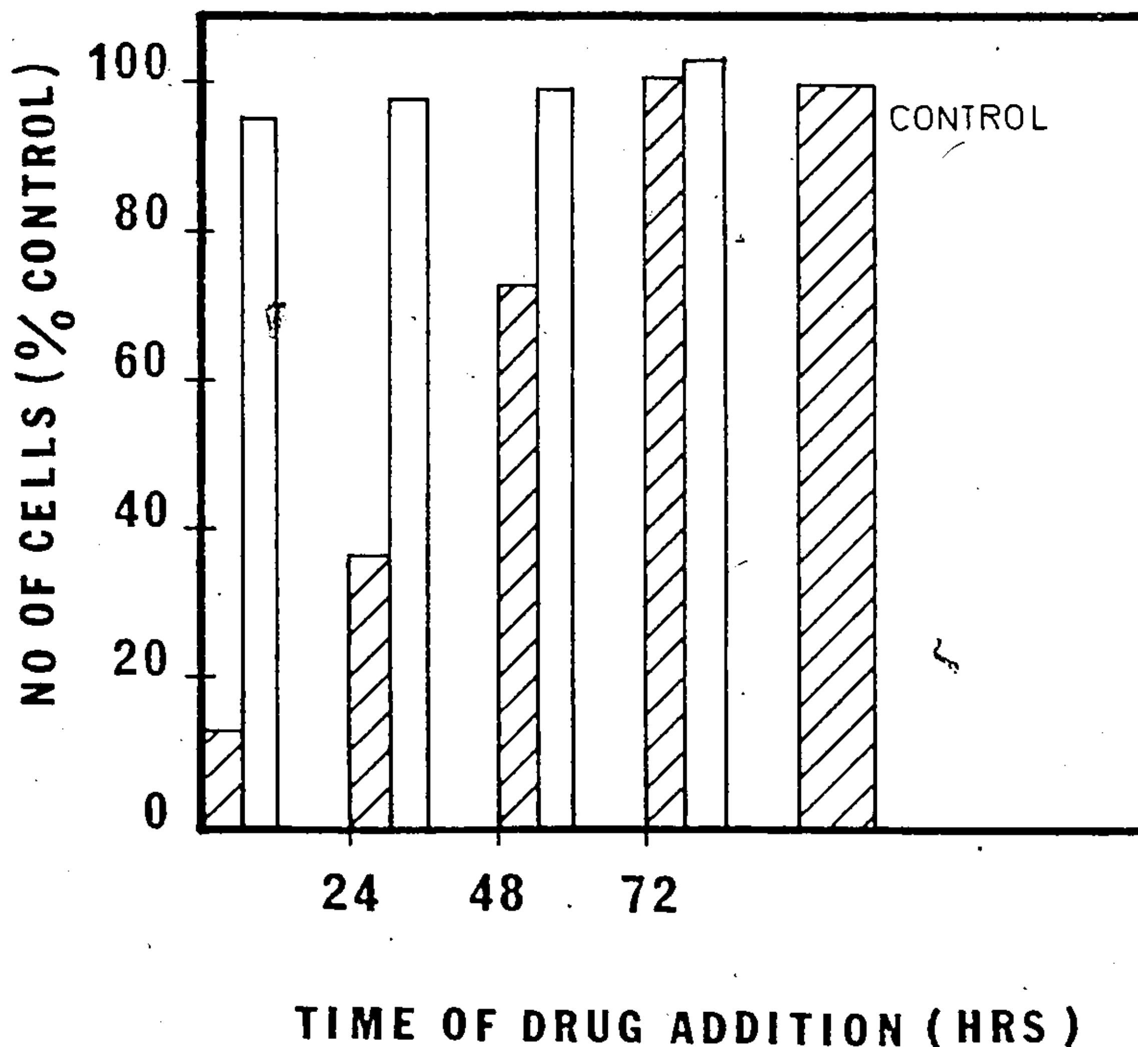



Figure 9: Effect of cyclophosphamide at various stages of LS2 cell growth.
0.1 ml of $10^{-1}$M cyclophosphamide was added to 9.9 ml of LS2 cells at 0, 24, 24 and 72 hours from the time the cells were subcultured. Control cells were grown without the drug. The total number of cells were counted before the drug was added (▨), at 0, 24, 48 and 72 hours. The cells were allowed to grow until the control cells reached the resting phase (4 days). Total number of cells were counted (▭) and the results were expressed as the percentage of the control cells. Each flask was set in triplicate.

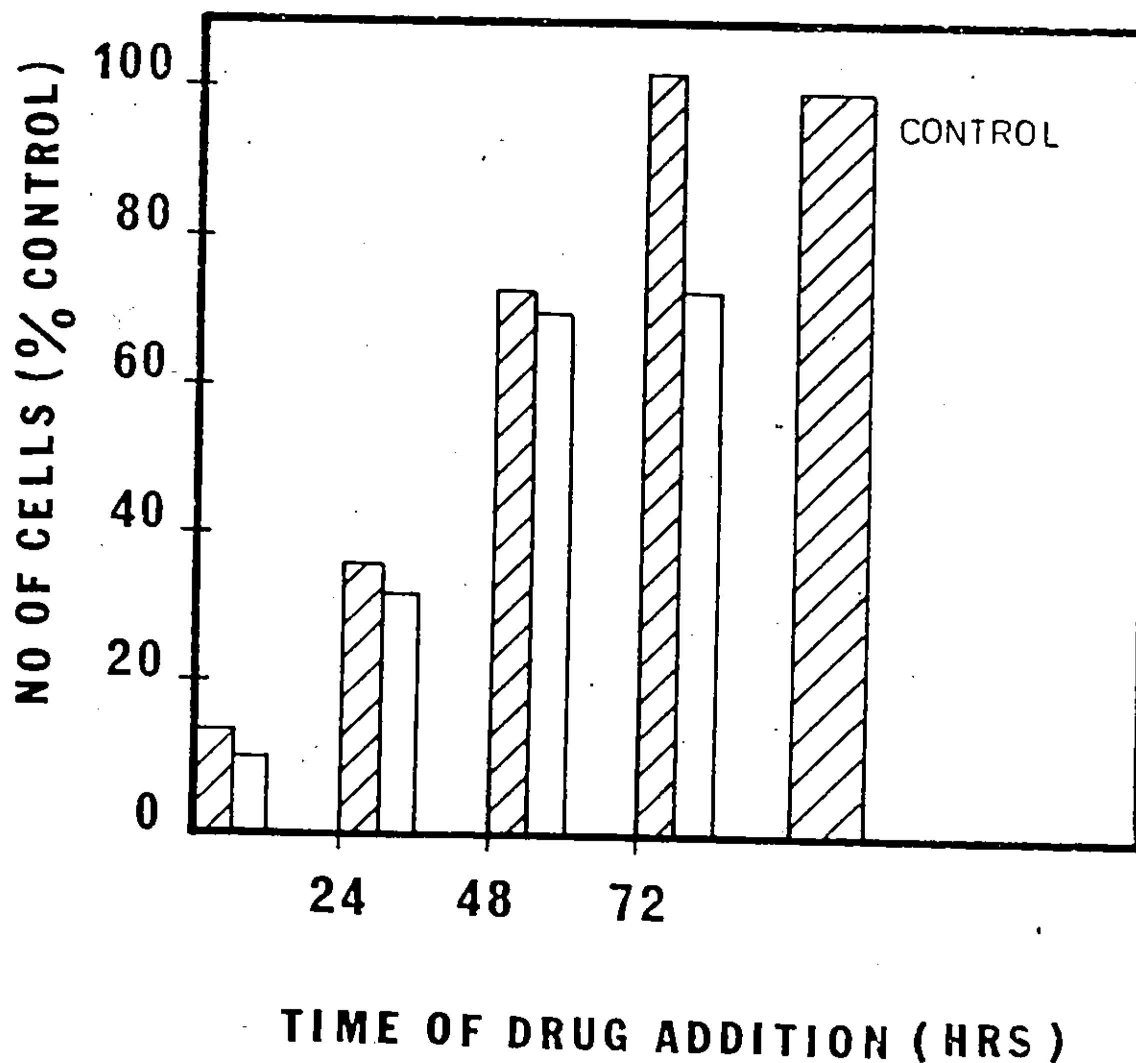



Figure 10: <u>Effect of phosphorodiamidic acid at various stages of LS2 cell growth.</u>
0.1 ml of $10^{-2}$M of phosphorodiamidic acid was added to 9.9 ml of LS2 cells at 0, 24, 24 and 72 hours from the time the cells were subcultured. Control cells were grown without the drug. The total number of cells were counted before the drug was added (▨), at 0, 24, 48 and 72 hours. The cells were allowed to grow until the control cells reached the resting phase (4 days). Total number of cells were counted (▭) and the results were expressed as the percentage of the control cells. Each flask was set in triplicate.

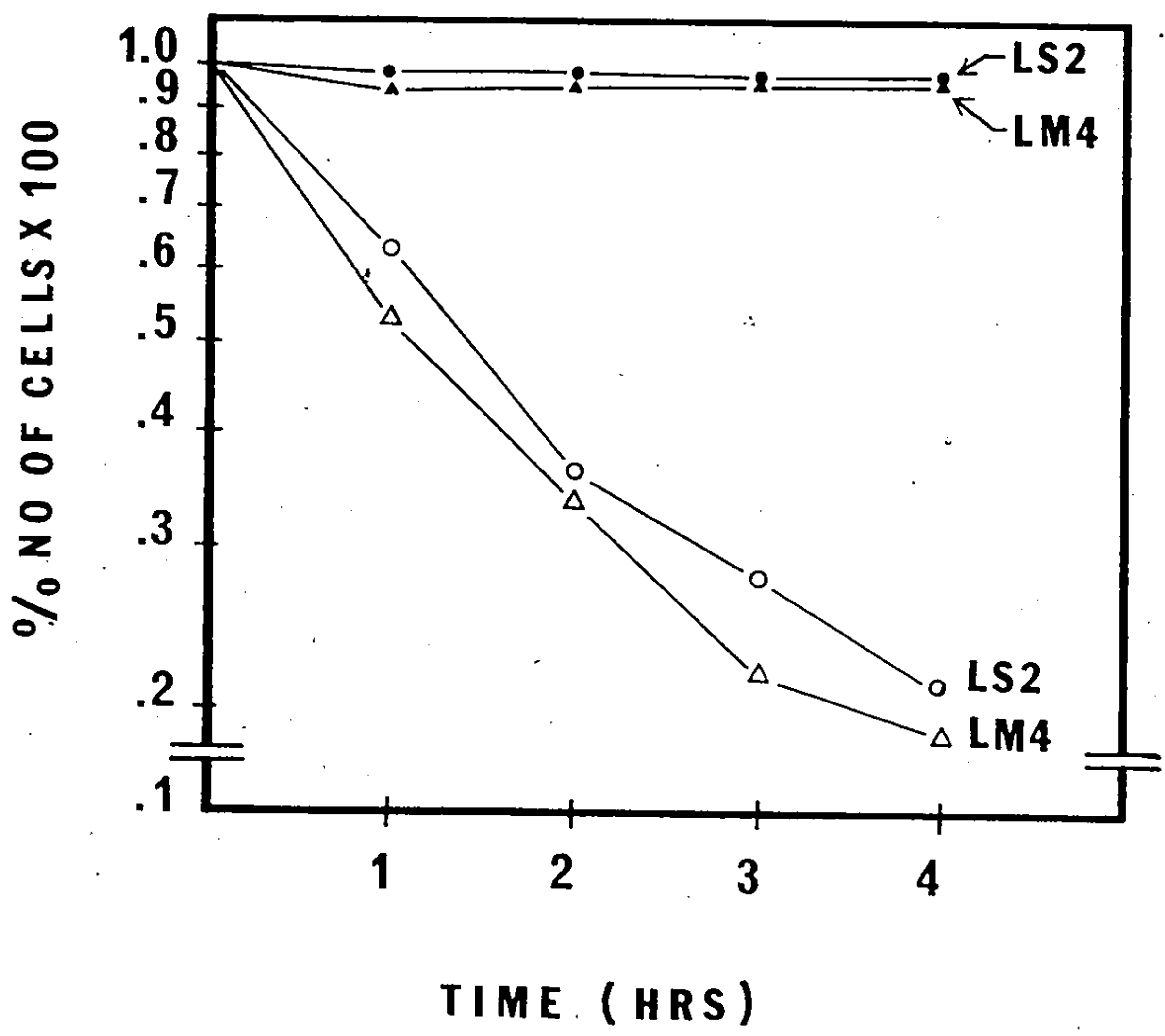
did not depend on the age of the cells, since no inhibition of growth was seen. Almost 100% inhibition was observed when the cells growing for 24 and 48 hours were treated with phosphorodiamidic acid (Figure 8 and 10). However, when phosphorodiamidic acid was added to cultures at 0 time or those grown for 72 hours, the effect was greater than 100%, suggesting that resting cells were actually killed by the drug, whereas the growth of log phase cells was inhibited. For subsequent experiments cells growing for 24 hours were used, to ensure that most of the cells were in log phase.

Phosphorodiamidic acid is unstable in water, 30% decomposing in seven hours (data supplied along with the drug from National Cancer Institute). Hence the effect of varying the time of exposure to the drugs was investigated. Figure 11 shows that increasing the time of exposure of the cells to the drug gave increasing inhibition. For subsequent experiment the LM4 cells alone were exposed to the drugs for four hours, since the effect of cyclophosphamide or phosphorodiamidic acid on LS2 and LM4 cells was very similar. This time of exposure to phosphorodiamidic acid inhibited cell growth by 80%. Figure 11 suggests that LM4 cells were sensitive to phosphorodiamidic acid, so the viability of the cells treated with phosphorodiamidic acid and cyclophosphamide was measured by two methods, trypan blue exclusion and colony forming assays. The trypan blue test indicated that the ratio of the dead cells to live cells and the total number of cells remained constant when the cells were stained and counted at 0, 4, 24, 48 and 72 hours after treatment with phosphorodiamidic acid. The live cells in the population treated with phosphorodiamidic acid appeared to be

Figure 11: <u>Effect of cyclophosphamide and phosphorodiamidic acid at various time exposures.</u>

Cells were grown in the absence of drug for 24 hours prior to addition of 0.1 ml of $10^{-1}$M cyclophosphamide and 0.1 ml of $10^{-2}$M phosphorodiamidic acid. After 0, 1, 2, 3 and 4 hours incubation of LM4 and LS2 cells, the cultures were centrifuged at 600 x g for 5 minutes, washed with 5 ml of 1640 medium, the resuspended in 10 ml of 1640 medium containing 10% fetal calf serum. Controls were set up without the drug. The cells were counted on the fourth day. Each experiment was performed in triplicate. The results were expressed as the percentage of the control cells.

△——△, LM4 treated with phosphorodiamidic acid
O——O, LS2 treated with phosphorodiamidic acid
▲——▲, LM4 treated with cyclophosphamide
●——●, LS2 treated with cyclophosphamide



1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
% NO OF CELLS X 100
LS2
LM4
LS2
LM4
1
2
3
4
TIME (HRS)

twice the size of normal cells. When compared to control cells, those treated with cyclophosphamide showed a similar increase in number and normal size. Attempts to determine the viability of the drug-treated cells by colony forming assay were carried as described in the Reagents and Methods section. Flasks containing 50, 100, 250, 500, 1000, 5000 and 10,000 cells were prepared. An aliquot of 1 x $10^5$ cells killed by mitomycin were added to the suspension to assist colony formation. Flasks containing 2 x $10^6$ mitomycin-killed cells were set up as a control. The colonies began to appear after 10 days, and were counted after 15 days. No colonies were formed in flasks containing killed cells alone, or in flasks with 50, 100, 250 and 500 live cells. An average of 4, 12 and 32 colonies were found in flasks containing 1000, 5000 and 10,000 live cells respectively. The low cloning efficiencies of LM4 cells, only approximately 0.2-0.4% under the experimental conditions used, restricted the usefulness of this technique, because in the drug-treated cultures the number of viable cells would be even lower.

3.2. Metabolism of cyclophosphamide

When cyclophosphamide was incubated with washed liver microsomes and appropriate cofactors, the reaction product could be extracted with chloroform at pH 4.0. A control experiment was performed by incubating the microsomes with the cofactors for 45 minutes, then cyclophosphamide was added to the mixture. Immediately after addition of cyclophosphamide the mixture was extracted with chloroform. Thin layer chromatography comparison of the control incubation showed that cyclophosphamide was

the only substance extracted with chloroform. Qualitative analysis by thin layer chromatography of the acidic extract from the incubation mixture suggested that phosphorodiamidic acid was the product formed. Authentic phosphorodiamidic acid was used as a marker.

A second product of the incubation (acrolein) which was trapped in a trapping flask was also measured. Table 2 shows that the amount of phosphorodiamidic acid formed from liver microsomes of phenobarbital treated rats was about 6.5 times greater than that of normal rat and the amount of acrolein trapped about 5.5 times greater. Hence, for subsequent experiments phenobarbital treated rats were used.

3.2.1. Isolation and identification of metabolites : Acrolein produced during the incubation was trapped in a trapping flask. Quantitative determination of acrolein by fluorometric techniques showed that standard curve of acrolein concentration between 0.077 μmoles and 0.616 μmoles was linear. Thin layer chromatography of the chloroform extract containing the metabolites was performed using chloroform-ethanol (9:1). The products were detected by spraying with Epstein's reagent (NBP), alkylating compounds giving blue spots. This method depends upon the ability of alkylating agents to alkylate NBP to the quaternary pyridinium ion which is highly coloured in alkaline medium. The reaction is not specific and the colour formed in unstable. The spot with an Rf value of 0.50 was identified as unreacted cyclophosphamide. There was also material which was immobile in the solvent system, chloroform-ethanol (9:1). The silica gel at the origin of the preparative

Table 2. Microsomal incubation of cyclophosphamide

| Type of rat | Amount of acrolein trapped | Amount of Phosphorodiamidic acid extracted |
|---|---|---|
| normal | 0.062 ± 0.010 μmoles | 0.012 ± 0.002 μmoles |
| phenobarbital treated | 0.388 ± 0.059 μmoles | 0.079 ± 0.008 μmoles |

Sodium phenobarbital was introduced into the rats drinking water at a level of 500 mg/litre, 10 days prior to the experiments. The experiments were done in duplicate and the values are the average of two experiments.

plate was scraped and eluted with methanol, evaporated to dryness and the residue dissolved in 1 ml MeOH. 100 µl of the methanolic solution was methylated with ethereal diazomethane (Struck et al., 1970). On TLC the reaction mixture showed three blue spots which gave mass spectra as in Figure 12, 13, and 14 identical with that reported as the mono, di and trimethyl ester of phosphorodiamidic acid (Fenselau et al, 1975) as well as the pure phosphorodiamidic acid derivatives. The rest of the methanolic solution was evaporated to dryness and diluted in 1 ml of distilled water. This solution was used for quantitative determination using Epstein's colorimetric determination, described in the Reagents and Methods. The colour formed is rather unstable, decreasing by 9% of the absorbance reading in 5 minutes. However, if the operation from the introduction of NaOH to the reading in the colorimeter was carried out rapidly and in the absence of direct light, the standard deviation of five samples used in different experiments, varied from 0.7 - 2.8%.

Table 3 shows the amount of acrolein and phosphorodiamidic acid produced during incubation of cyclophosphamide with liver microsomes and appropriate co-factors. The amount of acrolein produced was greater than the amount of phosphorodiamidic acid. The difference could be explained by the difference in their efficiencies of extraction. The low extraction efficiency of phosphorodiamidic acid as well as the low trapping efficiency of acrolein under the experimental conditions used restricted the accuracy of the results. The results confirmed that incubation of cyclophosphamide with liver microsomes produced acrolein

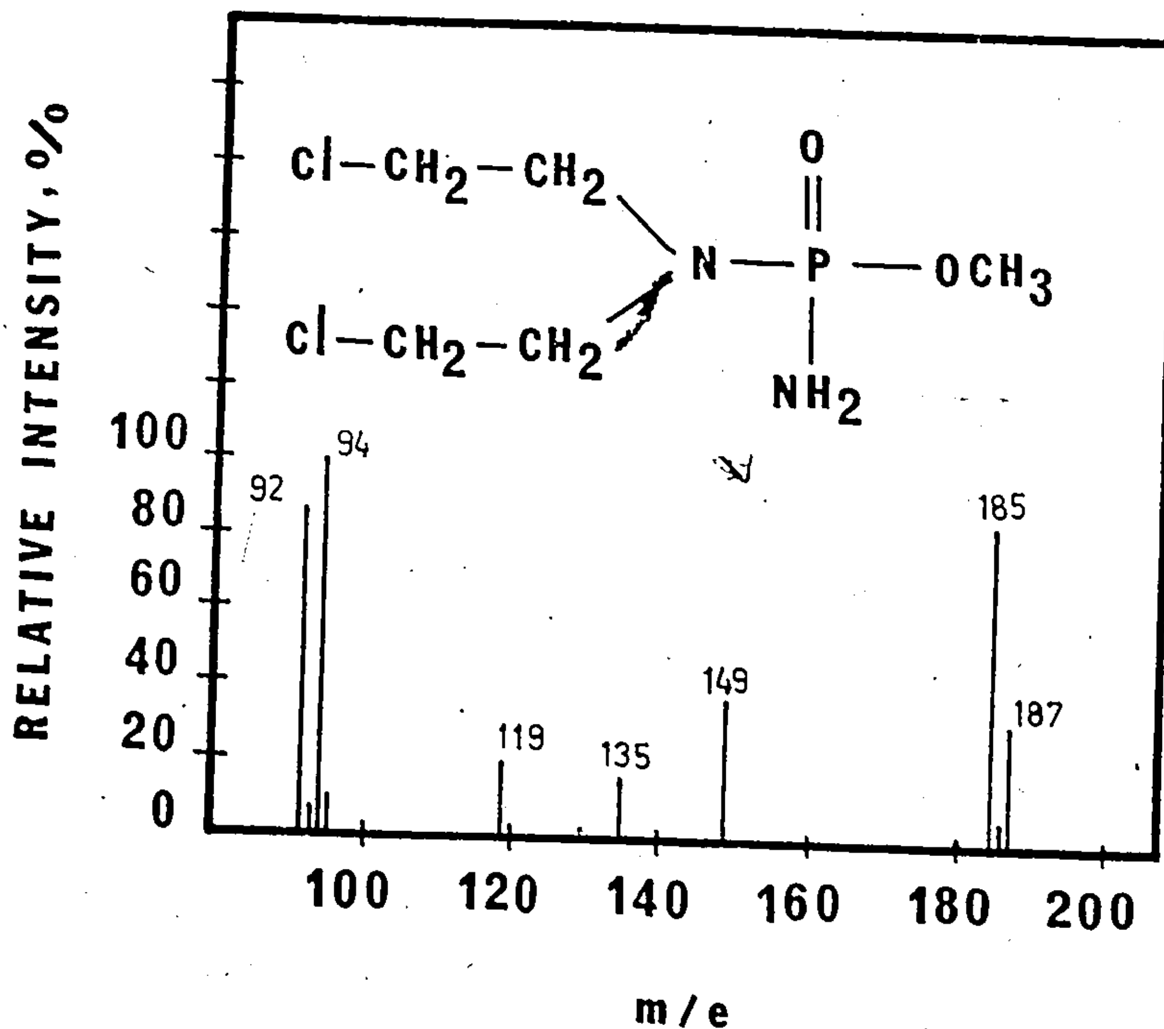



Figure 12: Mass spectral data of monomethyl derivative phosphorodiamidic acid.

An extract of phosphorodiamidic acid was reacted with diazomethane to produce derivatives suitable for gas chromatographic purification and mass spectral analysis. The reaction leads to a mixture of three products.

A. monomethyl derivative of phosphorodiamidic acid

B. dimethyl derivative of phosphorodiamidic acid

C. trimethyl derivative of phosphorodiamidic acid

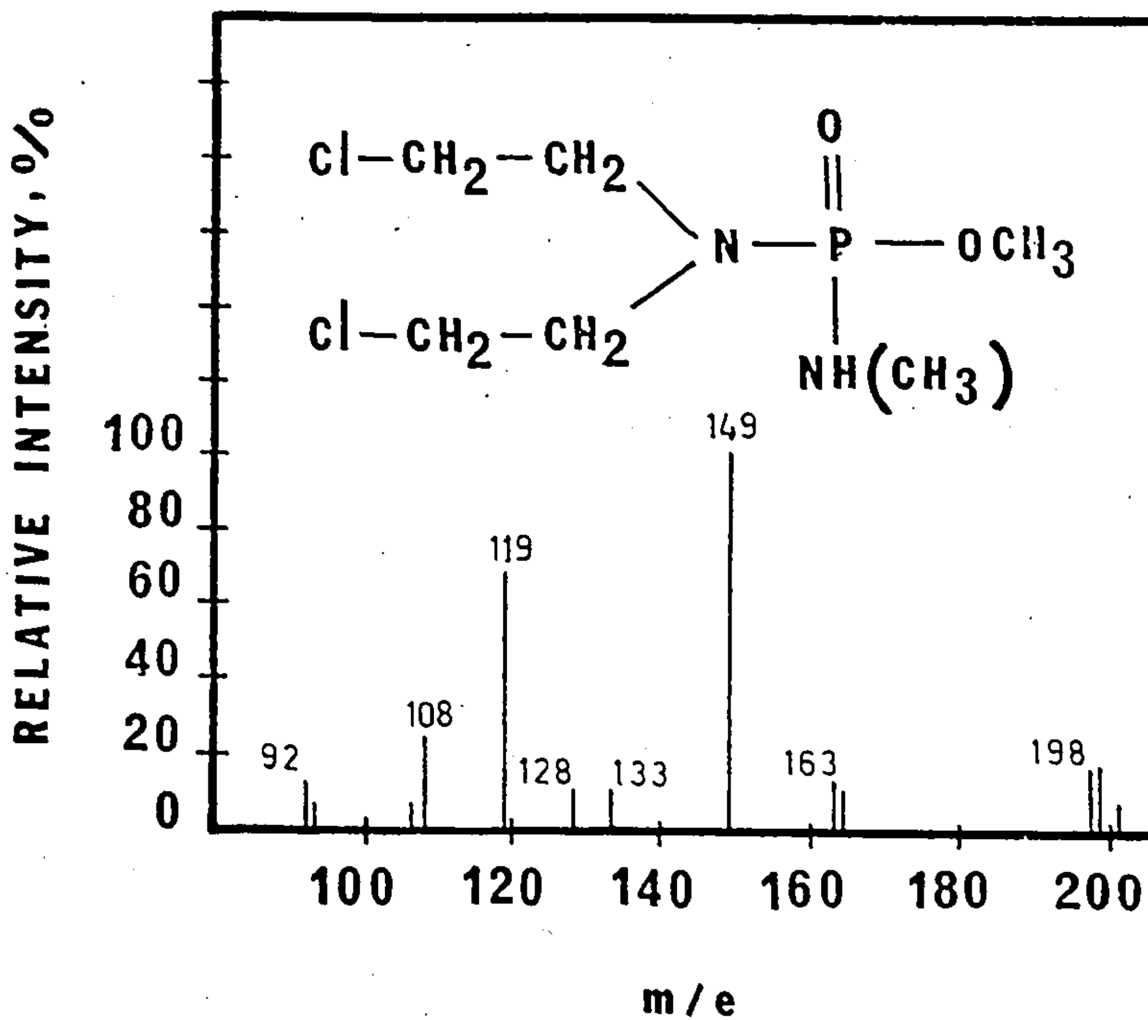



Figure 13: Mass spectral data of dimethyl derivative of phosphorodiamidic acid.

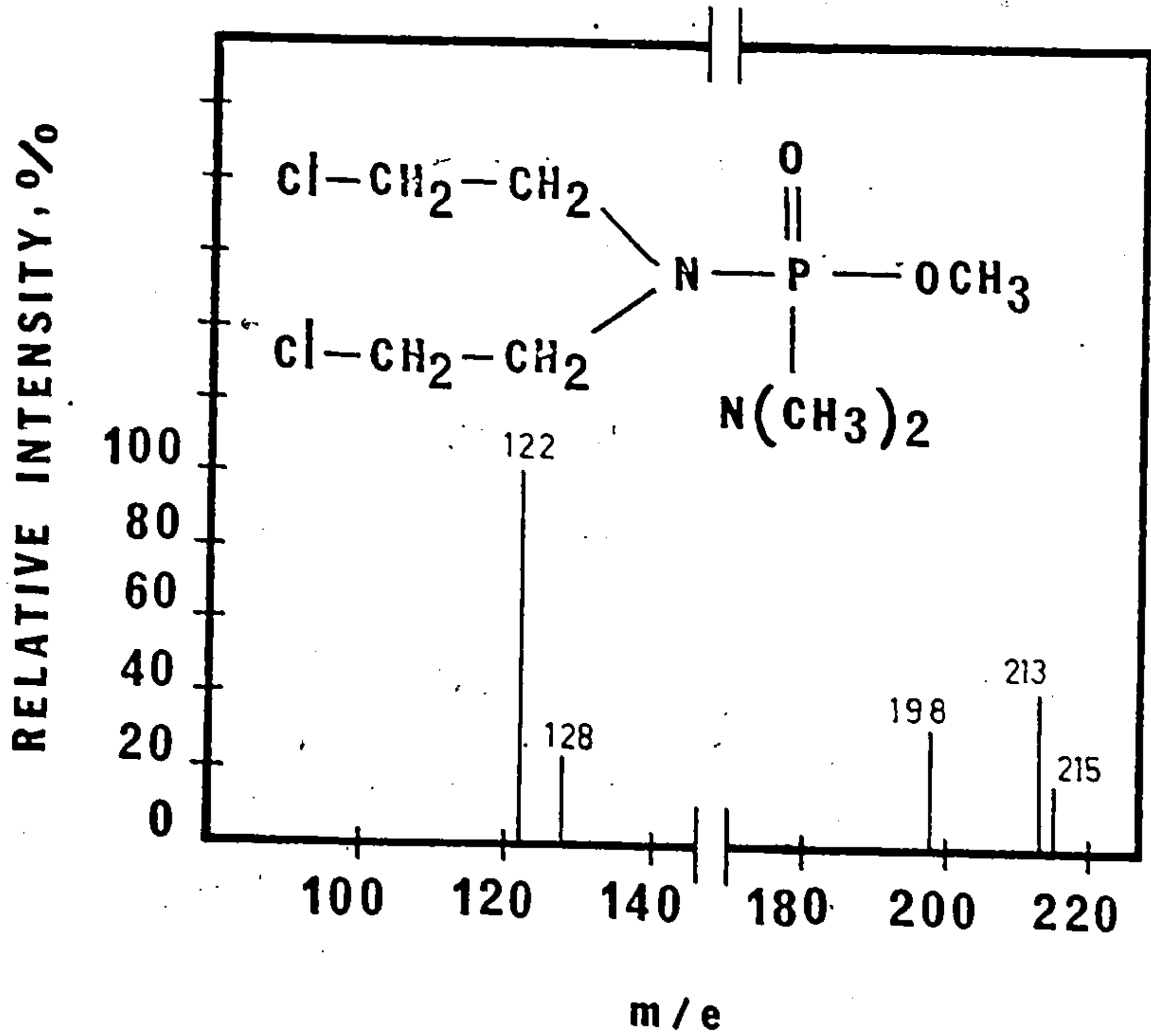



Figure 14: Mass spectral data of trimethyl derivative of phosphorodiamidic acid.

Table 3. Amount of materials produced during microsomal incubation of cyclophosphamide

| Sample | Amount of acrolein formed (μmoles) | Amount of phosphorodiamidic formed (μmoles) | % of acrolein trapped | % of phosphorodiamidic acid extracted |
|---|---|---|---|---|
| 54 μmoles of cyclophosphamide added | 0.55 ± 0.02 | 0.127 ± 0.013 | - | - |
| 30.8 μmoles acrolein added | 2.40 ± 0.21 | - | 7.79 ± 0.68 | - |
| 1.88 μmoles phosphorodiamidic acid added | - | 0.024 ± 0.012 | - | 1.27 ± 0.64 |

All the flasks contained the same amounts of microsomes and cofactors in a total volume of 30 ml buffered at pH 7.4 with 0.1 M Tris-HCl, incubated at 37°C for 45 minutes. All flasks were in duplicate. Five experiments were done for the first sample and two experiments for the second and third samples.

Total amount of acrolein produced = 7 ± 0.87 μmoles

Total amount of phosphorodiamidic acid produced = 10 ± 6 μmoles

and phosphorodiamidic acid, but did not prove that the amount of acrolein produced was equal to the amount of phosphorodiamidic acid. Attempts to improve the sensitivity and specificity of quantitation of phosphorodiamidic acid extract were not successful. When phosphorodiamidic acid extracts were treated with diazomethane to produce derivatives suitable for gas chromatographic and mass spectral analysis, the reaction lead to formation of mixture of three products, carrying one, two and three methyl groups. These methylated derivatives can be analyzed by gas chromatography-mass spectrometer but the presence of three different products limited the usefulness of this method for quantitation purposes. An attempt to develop a more uniform reaction was made using the alkylative extraction method (Ervik and Gustavii, 1974). Identification of the product by gas chromatography with flame ionization detector showed the apparent formation of a monomethyl derivative of phosphorodiamidic acid, but gas chromatography mass spectrometer analysis was not able to confirm this. Reagents used and by-products of the reaction seemed to interfere with the analysis of the product by gas chromatography-mass spectrometer during transmission across the Watson-Biemann separator. Since rigorous identification was difficult, the attempt was abandoned.

3.2.2. Determination of the stability of phosphorodiamidic acid and cyclophosphamide in incubation medium: The stability of phosphorodiamidic acid and cyclophosphamide when incubated with LM4 cells in medium containing 10% fetal calf serum was determined by thin layer chromatography. Phosphorodiamidic acid was extracted from the incubation

medium before being spotted on a thin layer plate. The results indicated that for four hours the alkylating activity of cyclophosphamide and phosphorodiamidic acid was unchanged qualitatively, since identical blue spots were observed for all the samples taken at half-hourly intervals. However, this method by no means indicated the quantity of the alkylating agents present at the different time intervals. Quantitation of the alkylating agents was not attempted because of the difficulties discussed earlier.

### 3.3. Intranuclear reactivity of cyclophosphamide and phosphorodiamidic acid in LM4 cells

The data in Table 4 showed that the biphasic partition method is suitable for separating double-stranded DNA from single-stranded DNA. Thus on average, 79% of native DNA migrated to the upper layer of the biphasic system during the first extraction. When DNA solution was made alkaline and then neutralized, an average of 12.3% of the DNA was present in the upper layer at the first extraction. Most of this DNA is regarded as spontaneously renaturable for whatever reason, and each subsequent extraction removed 1.2% of the input DNA into the upper layer.

When LM4 cells grown for 24 hours were treated with $1 \times 10^{-4}$M phosphorodiamidic acid for 4 hours and the DNA isolated, made alkaline and incubated at 37°C for 10 minutes, neutralized and extracted, the amount of DNA that migrated into the upper layer increased (Table 5). Student t test analysis indicated that the difference between amount of DNA extracted into the upper phase of phosphorodiamidic acid treated cells as compared

Table 4. Percentage of DNA extracted to the upper phase by biphasic partition system

| Extraction number | Denatured by alkaline and reneutralized | Not denatured |
|---|---|---|
| 1 | 12.25% ± 1.12% | 79.85% ± 1.52% |
| 2 | 1.25% ± 0.05% | |
| 3 | 1.20% ± 0.06% | |

Each sample was done in duplicate two experiments were done for all the samples.

Table 5. Estimation of cross-linking of DNA from intact cells incubated with $1 \times 10^{-4}$M phosphorodiamidic acid and $1 \times 10^{-3}$M cyclophosphamide

| | Control | Cyclophosphamide treated | Phosphorodiamidic acid treated |
|---|---|---|---|
| Concentration of DNA, µg/ml | 564 ± 26 | 556 ± 16 | 521 ± 13 |
| protein conc. in µg/ml | 20 ± 1 | 13 ± 0.6 | 16 ± 0.9 |
| concentration of RNA, µg/ml | negligible | negligible | negligible |
| % of DNA in upper phase | *14.66% ± 0.47% | 15.64% ± 1.90% | 30.85% ± 3.0% |

Each sample was done in duplicate, three experiments being carried out for all the samples. Efficiency of extraction of the double-stranded DNA into the upper phase was determined for each sample. The % of DNA extracted into upper phase was corrected to 100% of extraction efficiency. The extraction efficiency range from 70.5% ± 1.7% to 88.56% ± 2.04%.

* Mean ± standard deviation.

to control was highly significant. Cyclophosphamide, however, which had no effect on the growth of LM4 cells, showed no significant increase in the amount of DNA which was extracted into the upper layer, confirmed by the student t test analysis. Thus, DNA from cells treated with phosphorodiamidic acid but not cyclophosphamide showed a significant degree of cross-linking of the DNA.

Nuclei were isolated from LM4 cells pre-labelled with $^3$H-TdR and grown for 24 hours. The nuclei (400 μg DNA) were treated with $1 \times 10^{-3}$M cyclophosphamide or $1 \times 10^{-4}$M phosphorodiamidic acid for 4 hours, no drugs being added to the control flask. At the end of the incubation, unlabelled nuclei were added and DNA was isolated from each sample. The isolated DNA was treated as above and extracted in the phase system. Table 6 shows that the amount of cross-linked DNA after treatment with cyclophosphamide increased almost two-fold, but treatment with phosphorodiamidic acid remained relatively the same, as compared to incubation of the intact cells with the drugs. The student t test indicated that the amount of DNA extracted into the upper phase of cyclophosphamide or phosphorodiamidic acid treated was significant as compared to that from untreated nuclei, whereas there was no significant difference between amounts of DNA extracted into the upper phase of phosphorodiamidic acid and cyclophosphamide treated nuclei.

Figure 6. Estimation of cross-linking of DNA from nuclei incubation with $1 \times 10^{-4}$M phosphorodiamidic acid and $1 \times 10^{-3}$M cyclophosphamide

| | Control | CP treated | PD treated |
|---|---|---|---|
| Concentration of DNA μg/ml | 468 ± 16 | 402 ± 20 | 460 ± 14 |
| protein conc. μg/ml | negligible | negligible | negligible |
| RNA conc. μg/ml | negligible | negligible | negligible |
| % of DNA in upper phase | 16.31 ± 3.25 | 29.91% ± 3.50% | 32.17% ± 4.20% |

Each sample was done in duplicate, three experiments were done for all the samples. Efficiency of extraction of the double-stranded DNA into the upper phase was determined for each sample. The % of DNA extracted into upper phase was corrected to 100% of extraction efficiency. The extraction ranged from 80.9% ± 1.9% to 94.95% ± 3.23%.

## DISCUSSION

### 4.1. Effects of cyclophosphamide and phosphorodiamidic acid in intact cells

In agreement with previous reports by other workers (Maddock et al, 1966 and Connors et al, 1974b) our data demonstrate that phosphorodiamidic acid is at least 100 times more potent than cyclophosphamide as shown by the concentration required to inhibit the growth of LM4 and LS2 cells. Nevertheless cyclophosphamide is the more useful drug clinically since the more active phosphorodiamidic acid has greater toxicity and is less specific towards malignant tissue.

The growth inhibition we observed with LM4 and LS2 cells did not depend on the stage of growth of the cultures, since inhibition was obtained when phosphorodiamidic acid was added at different stages of cell growth, when the ratio of proliferating to non-proliferating cells varied. Cyclophosphamide, however, had no effect, as expected. The slightly greater killing effect observed when resting cells were treated with phosphorodiamidic acid may have resulted from the drug enhancing the cell death which occurs in depleted medium. This effect was not further investigated and cells in logarithmic phase were used for all subsequent experiments.

We found that exposure of cells to phosphorodiamidic acid caused enlargement of cells, in agreement with the general effect of alkylating agents. To confirm that the growth inhibition observed was a measure of

the cells killed, the trypan blue exclusion test was used to determine cell viability. Staining cells with vital dyes has been classically used as a criterion of cell death and as an indication of cell membrane damage (Hoskin et al, 1956; Phillips et al, 1957 and Shrek, 1936). Our results using this method were similar to those obtained in the experiments on inhibition of cell growth. However, a recent report by Ropen and Drewinko (1976) indicated that dye can be excluded from cells unable to reproduce as well as from living cells. Consequently, the results from the trypan blue experiments do not distinguish actual cell kill from a temporary lag in the multiplication rate. The study of Ropen and Drewinko (1976) also showed no correlation between the trypan blue test and the colony-forming ability of the cells. In our experiments, however, phosphorodiamidic acid inhibited growth completely and the live cells doubled in size, while cyclophosphamide showed no apparent effect on cell growth or size. It is therefore reasonable to conclude that phosphorodiamidic acid caused biochemical changes which led to lethally damaged cell, which can continue synthesizing protein and nucleic acid though they could not divide. In an attempt to confirm that this effect represented lethal damage to the cells, a colony-forming assay was attempted. The cloning efficiency of LM4 cells was very low. It has been reported (MacPherson and Bryden, 1971) that cloning efficiency could be improved if mitomycin-C killed cells are added to the preparation. However, addition of killed cells to LM4 cells did not increase their cloning efficiency, consequently no conclusion could be drawn from these data.

The cytotoxic effect of cyclophosphamide or phosphorodiamidic acid *in vitro* is directly proportional to the alkylating activity of the compounds under physiological conditions of pH and temperature, which in turn depends on the stability of the compounds. Phosphorodiamidic acid is rather unstable in aqueous medium, consequently its decomposition into non-alkylating agents during the incubation will decrease its cytotoxic effect. Studies on the cytotoxicity of cyclophosphamide and phosphorodiamidic acid as a function of time indicated that 4 hours of incubation with phosphorodiamidic acid resulted in 80% growth inhibition, while no effect was observed with cyclophosphamide (Figure 11). Alkylating activity of phosphorodiamidic acid and cyclophosphamide during the 4 hours incubation was determined qualitatively, and our results indicated that after 4 hours of incubation some alkylating activity of phosphorodiamidic acid was retained. Hohorst et al, (1976) reported that the alkylation reaction of phosphorodiamidic acid and water reached a maximum of 70% at 4 hours. Although some phosphorodiamidic acid may have decomposed into non-alkylating material, nevertheless, 80% inhibition of cell growth was reached after 4 hours of incubation. Cyclophosphamide on the other hand, showed no effect on the cell growth although its potential alkylating activity was still retained.

4.2. Metabolism of cyclophosphamide

4.2.1. Microsomal activation in vitro: When cyclophosphamide was incubated with washed microsomes and the appropriate cofactors, phosphorodiamidic acid and acrolein were generated, 4-hydroxycylophosphamide and

aldophosphamide are too unstable to allow their identification by the method employed. Phosphorodiamidic acid was identified by thin layer chromatography and gas chromatography-mass spectrometry after reaction with diazomethane. We obtained spots of identical Rf values and the same mass spectral data as given by the pure material and that reported by Fenselau et al, (1975). The present results are thus consistent (see Figure 1) with metabolic activation of cyclophosphamide. It is well established that activation of cyclophosphamide involves the mixed function oxidase enzyme in the liver microsomes, hence pretreatment of the liver with phenobarbital will potentiate the activation. Our results confirm that pretreatment of the rats with phenobarbital prior to the experiment increased the production of metabolites. Sladek (1972a) found a three-fold increase of the alkylating activity in the blood of rat treated with phenobarbital for 5 days. In our studies, five-to six-fold increases were observed, perhaps because of longer periods of treatment with phenobarbital.

4.2.2. Quantitative determination of cyclophosphamide metabolites: If phosphorodiamidic acid is the final active metabolite which alkylates as an intact molecule, the alkylating effect of phosphorodiamidic acid generated from *in vitro* microsomal activation of cyclophosphamide should be the same as the effect of equal amounts of pure phosphorodiamidic acid. The scheme in Figure 1 (see Introduction) implies that the amount of acrolein and phosphorodiamidic acid formed should be equal. Taking into account the percent of recovery of each, the total amount of acrolein

produced was not equal to the amount of phosphorodiamidic acid produced. It is difficult to draw any conclusion from these results, mainly because the present experimental procedure lacks reproducibility and sensitivity, especially in the recovery of phosphorodiamidic acid in experiments performed on separate occasions. However, the reproducibility of isolation and quantitation of phosphorodiamidic acid produced during activation of cyclophosphamide was better. Colvin et al, (1973) reported that 46% of alkylating activity in the supernatant was generated from microsomal incubation of cyclophosphamide in 30 minutes (measured by the NBP assay with nor-nitrogen mustard as standard). Our data, however, demonstrate a much lower amount of phosphorodiamidic acid generated. In their report, these workers determined total alkylating activity in the supernatant, which contained unreacted cyclophosphamide as well as other metabolites which have alkylating activity. Cyclophosphamide reacts with 4-p-nitrobenzylpyridine, although to a lesser extent. Sladek (1972a) indicated that cyclophosphamide has about 2.5% the alkylating activity of nor-nitrogen mustard on a molar basis. This may be the reason for the discrepancy between these results.

Given the difficulties with available methods, a quantitative assay for phosphorodiamidic acid with high specificity and sensitivity by gas chromatography-spectrometry was attempted. Fenselau et al, (1975) have identified phosphorodiamidic acid by selected ion monitoring on gas chromatography-mass spectrometry after derivatization of phosphorodiamidic acid with diazomethane. Gas chromatography-mass spectrometry is a higly specific and sensitive analytical technique (Fenselau, 1974).

Unfortunately reaction of phosphorodiamidic acid with diazomethane produced three products in varying ratios, and so was unsuitable.for quantitative determination. During the preparation of this manuscript Jardine et al, (1976) have synthesized tetradeuterated phosphorodiamidic acid which could be used as an internal standard, and quantitation of phosphorodiamidic acid by the method of Fenselau et al, (1975) is now possible using this internal standard and so monitoring one of the derivatives. The extractive alkylation method used for derivatization of phosphorodiamidic acid results in the apparent production of the monomethyl derivative, identified by gas chromatography. Positive identification of this product by gas chromatography-mass spectrometry was not successful due to interference caused by other products of the reactions. Attempts to separate the product from the contaminants were not successful, due to the polar nature of the derivative and contaminants. The formation of a single derivative by extractive alkylation could be useful in quantitation of phosphorodiamidic acid if purification of the product is possible. The assay for acrolein was sensitive but the trapping system was not adequate. Modification of the trapping flask was attempted but without success.

## 4.3. Reactivity of cyclophosphamide and phosphorodiamidic acid with DNA on intact cells and nuclei

In agreementwith previous reports by other workers (Albert, 1967 and Walker, 1971), our data demonstrate that the biphasic partition method is suitable for separating double-stranded DNA from single-stranded

DNA, based on the rapid renaturation of the cross-linked molecule. When phosphorodiamidic acid was incubated with intact cells, and their DNA denatured, rapidly renatured and extracted, significantly higher amounts of double-stranded DNA were extracted into the upper phase as compared to control, while cyclophosphamide treated cells showed no difference from controls. Based on these observations we conclude that phosphorodiamidic acid can cross-link DNA but cyclophosphamide cannot. The fifteen percent of double-stranded DNA extracted into the upper phase in control and cyclophosphamide treated cells may represent some spontaneous renaturation of DNA or perhaps intrastrand double helix formed from the single-stranded DNA. This is the first report that phosphorodiamidic acid is able to cross-link the strands of DNA. This further strengthens its role as the active metabolite of cyclophosphamide.

The incubation of nuclei with cyclophosphamide with ten times as much DNA as was present in the intact cells under the same conditions resulted in two fold increase in the amount of cross-linked DNA, while the high degree of cross-linking in DNA was unchanged when nuclei were under the same conditions with phosphorodiamidic acid. The nuclei were exposed to higher concentration of drugs compared to the total amount of drug taken up by the intact cell, but this effect was partially compensated by increasing the concentration of nuclei to give ten times the amount of DNA. If increasing the amount of DNA available for alkylation caused an increase in the amount of DNA alkylated , the amount of DNA alkylated by phosphorodiamidic acid should have increased proportionately. Perhaps the degree of cross-linking of DNA has a maximum value, beyond which

breakage of DNA molecule may occur (e.g. a depurination effect of the alkylating agent). No experiment was done to check this. Walker (1971) found that the amount of DNA that migrated into the upper layer increased in a linear fashion at low concentrations of mustard gas, but his data (Table 7) indicate saturation at higher concentrations. Unpublished results of Harrap et al, showed about 33% of DNA was present in the upper phase after treatment with chloroambucil of animals bearing Yoshida sarcoma cells resistant to the drug and rapidly declining to 6%. The sensitive cells reached maximum of 20% at a slower rate but this remained constant. Our maximum was 32% is in the range of these reports.

If the unesterified hydroxyl group of phosphorodiamidic acid is important in alkylation, as suggested by Colvin et al, (1976), the intact molecule of cyclophosphamide would not be able to alkylate DNA, unless it was first activated. No evidence has been shown that nuclei are capable of activating cyclophosphamide. If cyclophosphamide itself is capable of alkylating DNA, this is the first report of such an effect.

The cross-linking observed was based on <u>in vitro</u> experiments using high concentrations of cyclophosphamide and phosphorodiamidic acid, much in excess of those which produce pharmacological effects in whole animal. Thus, it is difficult to draw conclusions of direct pharmacological significance from our results. Further studies are required to determine whether cyclophosphamide as an intact molecule can alkylated DNA or only after activation in the nucleus. Cyclophosphamide labelled in the ring and the side chain would be useful in such a study so that the fate of the label could be followed.

In conclusion, our studies have demonstrated that (a) in intact cells, phosphorodiamidic acid but not cyclophosphamide influences the growth and volume of the cells, which may lead to lethal damage to the cells; (b) phosphorodiamidic acid and acrolein are produced during microsomal activation of cyclophosphamide and the reaction could be potentiated by pretreatment of the rat with phenobarbital; (c) phosphorodiamidic acid and acrolein could not be measured accurately, hence, the stoichiometry of the conversion could not be determined; (d) phosphorodiamidic acid is capable of alkylating DNA in intact cells and of producing detectable cross-linking of DNA, but cyclophosphamide is not; both cyclophosphamide and phosphorodiamidic acid are capable of alkylating DNA when incubated with nuclei.

BIBLIOGRAPHY

Alarcon, R.A. (1968). Anal. Chem. 40, 1704-1708.

Alarcon, R.A. and Meienhofer, J. (1971). Nature New Biol. 233, 250-252.

Alberts, B.M. (1967). Biochem. 6, 2527-2532.

Alberts. B.M. (1968). Methods in Enzymology XLL, part A, 566-581.

Arnold, H. and Bourseaux, F. (1958). Angew. Chem. 70, 539.

Arnold, H., Bourseaux, F. and Brock, N. (1958). Naturwissenschaften 45, 64.

Bagley, C.M., Jr., Bostock, F.W. and De Vita, V.T. (1973). Cancer Res. 33. 226-233.

Brock, N., Gross, R., Hohorst, H.J., Klein, H.O. and Schneider, B. (1971). Cancer 27, 1512-1529.

Brock, N. (1976). Cancer Treat. Rep. 60, 301-308.

Burton, K. (1956). Biochemical J. 62, 315-323.

Calabresi, P. and Parks, R.E. (1976). The Pharmacological Basis of Therapeutic, 5th ed., 1254-1268, The MacMillan Company, New York.

Casperson, T., Farber, S., Foley, G.E., Killander, D. and Zetterberg, A. (1965). Exp. Cell Res. 39, 365-385.

Cline, M.J.,and Haskell, C.M. (1975). Cancer Chemotherapy, 2nd ed., 28-31.

Chun, E.H.L., Gonzales, L., Lewis, F.S., Jones, J. and Rutman, R.J. (1969). Cancer Res. 29, 1184-1194.

Cohen, J.L. and Jao, J.Y. (1970). J. Pharmacol. Exp. Ther. 174, 206-210.

Colvin, M., Padgett, C.A. and Fenselau, C. (1973). Cancer Res. 33, 915-918.

Colvin, M., Brundrett, R.B., Kan, M.N.N., Jardine, I. and Fenselau, C. (1976). Cancer Res. 36, 1121-1126.

Connors, T.A., Grover, P.L. and Mcloughlin, A.M. (1970). Biochem. Pharmacol. 19, 1533-1535.

Connors, T.A., Cox, P.J., Farmer, P.B., Foster, A.B., Jarman, M. and MacLeod, J.K. (1974a). Biomed. Mass Spectrometry 1, 130-136.

Connors, T.A., Cox, P.J., Farmer, P.B., Foster, A.B. and Jarman, M. (1974b) Biochem. Pharmacol. 23, 115-129.

Cox, P.J., Farmer, P.B. and Jarman, M. (1975). Biochem. Pharmacol. 24, 599-606.

Cox, P.J. and Levin, L. (1975). Biochem. Pharmacol. 24, 1233-1235.

Cox, P.J. and Farmer, P.B. (1975). Chem. Biol. Interaction. 10, 103-114.

Cox, P.J., Phillips, B.J. and Thomas, P. (1976a). Cancer Treat. Rep. 60, 321-326.

Cox, P.J., Farmer, P.B., Foster, A.B., Gilby, E.D., and Jarman, M. (1976b). Cancer Treat. Rep. 60, 483-491.

Draeger, U., Peter, G. and Hohorst, H.J. (1976). Cancer Treat. Rep. 60, 355-359.

Draeger, U. and Hohorst, H.J. (1976). Cancer Treat. Rep. 60, 423-427.

Ervik, M. and Gustavii, K. (1974). Anal. Chem. 46, 39-42.

Fenselau, C. (1974). Appl. Spectrometry 28, 305-318.

Fenselau, C., Kan, M.N.N., Billets, S. and Colvin, M. (1975). Cancer Res. 35, 1453-1457.

Foley, G.E., Friedman, O.M. and Drolet, B.P. (1961). Cancer Res. 21, 57-63.

Friedman, O.M. and Seligman, A.M. (1954). J. Amer. Chem. Soc. 76, 655.

Friedman, O.M. and Boger, E. (1961). Anal. Chem. 33, 906-910.

Friedman, O.M., Boger, E., Grubliauskas, V. and Sommer, H. (1963). J. Med. Chem. 6, 50-58.

Friedman, O.M., Wodinsky, I. and Myles, A. (1976). Cancer. Treat. Rep. 60, 337-346.

Goldenberg, G.J., Land, H.B. and Cormack, D.V. (1974). Cancer Res. 34, 3274-3282.

Goldenberg, G.J. (1975). Cancer.Res. 35, 1687-1692.

Graul, E.H., Schaumloffel, E., Hundeshagen, H., Wilmanns, H. and Simon, G. (1967). Cancer 20, 896-899.

Hill, D.L., Laster, W.R. and Struck, R.F. (1972). Cancer Res. 32, 658-665.

Hillcoat, B.L. (1971). J. Nat. Cancer Inst. 46, 75-80.

Hoskins, J.M., Meynell, G.G. and Sanders, F.K.A. (1956). Exptl. Cell Res. 11, 297-305.

Hohorst, H.J., Draeger, U., Peter, G. and Voelcker, G. (1976). Cancer Treat Rep. 60, 309-315.

Jardine, I., Brundrett, R., Colvin, M. and Fenselau, C. (1976). Cancer Treat Rep. 60, 403-408.

Jarman, M., Gilby, E.D., Foster, A.B. and Bondy, P.K. (1975). Clinica Chimica Acta 58, 61-69.

Jolley, G.M. and Ormeod, M.G. (1973). Biochemical et Biophysica Acta 308, 242-251.

Kirby, K.S. and Cook, E.A. (1967). Biochem. J. 104, 254-257.

Kohn, K.W., Steigbigel, N.H. and Spears, C.L. (1965). Proc. Natl. Acad. Aci. U.S. 53, 1154-1161.

Lawley, P.D. and Brookes, P. (1963). Biochem. J. 89, 127-138.

Lawley, P.D. (1966). Progr. Nucl. Acid Res. 5, 89-131.

Layde, J.P.,and Baserga, R. (1964). British J. of Cancer 18, 150-158.

Levis, A.G., Danieli, G.A. and Piccini, E. (1965). Nature 207, 608-619.

Ludlum, D.B. (1975). Antineoplastic and Immunosuppressive Agents, Part 11, 6-17, Sartorelli, A.C. and Johns, D.G. (Ed.), Springer Verlag, Berlin.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). J. Biol. Chem. 193, 265-241.

MacPherson, I. and Bryden, A. (1971). Exptl. Cell Res. 69, 240-241.

Maddock. C.L., Handler, A.H., Friedman, O.M., Foley, G.E. and Farber, S. (1966). Cancer Chemotherapy Rep. 50, 629-639.

Ohira, S., Maezawa, S., Irinoda, Y., Watanabe, K., Kitada, K. and Saito, T. (1975). Tohoku J. Exp. Med. 116, 341-350.

Peterson, A.R., Fox, B.W. and Fox, M. (1973). Biochemicia et Biophysica Acta 299, 385-396.

Phillips, H.J. and Terryberry, J.E. (1957). Extpl. Cell Res. 13, 341-347.

Phillips, H.J. (1973). Tissue Culture Methods and Application, Chap. 3, 406-408, Academic Press, New York.

Rickwood, D., Riches, P.G. and MacGillivray, A.J. (1973). Biochemica et Biophysica Acta 299, 162-171.

Roper, P.R. and Drewinko, B. (1976). Cancer Res. 36, 2182-2188.

Shrek, R. (1936). Am. J. Cancer 28, 389-397.

Sladek, N.E. (1971). Cancer Res. 31, 901-908.

Sladek, N.E. (1972a). Cancer Res. 32, 535-542.

Sladek, N.E. (1972b). Cancer Res. 32, 1848-1854.

Sladek, N.E. (1973). Cancer Res. 33, 651-658.

Struck, R.F., Kirk, M.C., Mellett, L.B., Dareer, S.E. and Hill, D.L. (1971). Mol. Pharmacol. 7, 519-529.

Struck, R.F. (1974). Cancer Res. 34, 2933-2935.

Struck, R.F., Kirk, M.C., Witt, M.H. and Laska, W.R., Jr. (1975). Biomed. Mass Spectrometry 2, 46-52.

Takamizawa, A., Tochino, Y. and Hamashima Y. (1972). I. Chem. Pharmacol. Bull. 20, 1612-1616.

Torkelson, A.R., LaBudde, J.A. and Weikel, J.H., Jr. (1974). Drug Metabolism Reviews 3, 131-165.

Van Durn, B.L. (1969). Ann. N.Y. Acad. Sci. 163, 589.

Voelcker, G., Draeger, U. and Peter, G. (1974). Arzneim Forsch 24, 1172-1176.

Voelcker, G., Wagner, T. and Hohorst, H.J. (1976). Cancer Treat. Rep. 60, 415-422.

Walker, I.G. (1971). Can. J. Biochem. 49, 332-336.

Warwick, G.P. (1963). Cancer Res. 23, 1315-1333.

Wheeler, G.P. (1973). Cancer Medicine, 791-806, Lea and Febiger, Philadelphia.

Wheeler, G.P., Bowden, B.J., Adamson, D.J. and Vail, M.H. (1969). Cancer Res. 30, 100-111.