STUDIES ON THE EFFECTS OF CYTOTOXIC AGENTS ON MOUSE THYMOCYTES IN VITRO

by

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ABSTRACT

There is at present no satisfactory method of predetermining the sensitivity of an individual human tumour to cancer chemotherapeutic agents. Using mouse thymocytes as a model cell system and the uptake of labelled metabolites (measured autoradiographically) as an indication of cell metabolism, the parameters of a possible *in vitro* sensitivity test have been investigated. The cytotoxic agents examined are all used for cancer chemotherapy. In general, the longer the alkylating agents were allowed to act on the thymocytes before the ability of the cells to incorporate thymidine-H^3 was assessed, the lower the thymidine-H^3 uptake. Because the extent of this enhanced drug effect after pretreatment varied from drug to drug, different drugs appeared to be the most effective inhibitors of thymidine-H^3 uptake by mouse thymocytes for each of the three incubation schedules used.

Evidence is presented which suggests that the pretreatment effect is not due solely to the alkylating agents causing a block at one point in the cell cycle, nor (for nitrogen mustard) is it due to a lag period before the damage becomes detectable. It is suggested that the pretreatment effect found in these short-term experiments is due to continuous and cumulative damage to the cells and that the extent of the pretreatment effect for each drug may be determined by the rate of reaction of that drug within the cells. Chlorambucil may be acting in a different manner from nitrogen mustard.

Since the uptake of uridine-H^3 and tritiated amino acids by the

thymocytes was reduced by drug concentrations very similar to those needed to inhibit uptake of thymidine-H^3, it is concluded that these drugs are not acting selectively against DNA synthesis. Evidence to support the view that nitrogen mustard is not acting selectively against cells in the S phase or reacting with the thymidine-H^3 is also presented. It appears likely that these alkylating agents are interfering with several metabolic pathways under the proposed experimental conditions and cause general cell death. The effects of these results on the clinical application of such an <u>in vitro</u> sensitivity test are discussed.

C O N T E N T S

Page

ABSTRACT 2

ACKNOWLEDGEMENTS 7

INTRODUCTION

Cytotoxic agents 8
Main groups of cancer chemotherapeutic agents . . 8
Alkylation: unimolecular mechanism; bimolecular mechanism; biological nucleophilic centres . . . 9
Main groups of alkylating agents: nitrogen mustards; ethyleneimines; epoxides; methanesulphonates . . 10
Action of alkylating agents in the cell 17
Drug action on nucleic acids 18
Drug action on proteins 21
Cell cycle block 25
Antimetabolite action 28
Use of alkylating agents for cancer chemotherapy . 32
Selectivity of drug action 33

EXPERIMENTAL PLAN

Cell system 37
Measurement of cell damage 40
Measurement of thymidine-H^3 incorporation . . . 43

EXPERIMENTAL WORK

Material and methods
Mice 46
Nutrient medium 46
Labelled metabolites 47
Cytotoxic agents 47
Diluents 48
Thymus cell suspensions 49
Incubation procedure 49
Autoradiographic procedure 50
Measurement of cell diameter 52
Estimation of alkylating radicals 52

Page

RESULTS

1. Uptake of labelled metabolites by untreated cells

Thymidine-H^3 uptake in the absence of cytotoxic agents 55
Correlation between cell size and ability to incorporate thymidine-H^3 57
Lack of effect on thymidine-H^3 uptake of mixing thymocytes from different mouse strains 59
Cell concentration 59
Thymidine phosphorylase 61
Duration of experiments 66
Uptake of tyrosine-H^3 and leucine-H^3 in the absence of cytotoxic agents 70
Correlation between cell size and uptake of tyrosine-H^3 and leucine-H^3 70

2. Effects of cytotoxic agents

Effect of nitrogen mustard on thymidine-H^3 uptake by mouse thymocytes 76
Comparative effects of several alkylating agents on thymidine-H^3 uptake 79
Cause of the pretreatment effect; time lag before the damage becomes detectable; continuous and cumulative damage 91
Effect of selected agents on uridine-H^3 uptake by mouse thymocytes in vitro: nitrogen mustard; melphalan; chlorambucil; mannitol myleran; summary of drug effects on uridine-H^3 uptake . 97

Page

Results: 2. Effects of cytotoxic agents (continued)

Effect of methotrexate on uptake of thymidine-H^3 and uridine-H^3 by mouse thymocytes *in vitro* . . . 105

Comparative effects of alkylating agents on tyrosine-H^3 uptake by mouse thymocytes *in vitro*: nitrogen mustard; thioTEPA; tretamine; mannitol myleran; melphalan; a possible antimetabolite effect shown by melphalan 109

Effect of nitrogen mustard on uptake of leucine-H^3 . 124

Preferential damage to S phase cells 125

Effect of nitrogen mustard on cell concentration . . 130

Effect of nitrogen mustard on thymocytes previously labelled with thymidine-H^3 130

DISCUSSION 136

REFERENCES 149

LIST OF APPENDIX TABLES 1 - 36 164

APPENDIX TABLES 1 - 36 167

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The two experiments described on page 63 were performed by Dr E. H. Cooper on mouse thymocyte suspensions prepared by me.

INTRODUCTION

Cytotoxic Agents

A cytotoxic agent can be defined as a substance (chemical or physical) capable of interfering with the normal metabolism of an organism in a manner harmful to the survival or reproduction of that organism. Cytotoxic agents which produce a selective effect in a diseased individual against the organism causing that disease can be used to advantage for chemotherapy. These undesirable organisms may be protozoa, fungi, bacteria, viruses or malignant cells. Although agents effective against many diseases of protozoal and bacterial origin are now available to the clinician, chemotherapy of viral-induced disease and of malignant tissue is very much in its infancy. The discussion and experimental work that follow have been limited to certain cytotoxic agents employed for cancer chemotherapy.

Main Groups of Cancer Chemotherapeutic Agents

Alkylating agents

Antimetabolites

Antibiotics and Alkaloids

Corticosteroids

Only the first group, the alkylating agents, will be discussed further in any detail.

Alkylation

Alkylation is the replacement of a hydrogen atom, H, in a molecule by an alkyl radical, R, or the addition of an alkyl radical to a molecule containing an atom in a lower valency state.

$$X{-}H \longrightarrow X{-}R$$

$$^{-}OOC{\cdot}CH_3 \longrightarrow ROOC{\cdot}CH_3$$

The alkyl radical, R, has the general formula C_nH_{2n+1} but the definition can be extended to include compound radicals such as $-CH_2CH_2COOH$. When detached from the rest of the alkylating agent molecule, the alkyl radical is positively charged, R^+, and can combine with an electron-rich centre, X^-. These electron-rich centres are described as nucleophilic and the positively charged alkyl radical as electrophilic. There are two main mechanisms by which the alkyl radical becomes transferred from the intact molecule of an alkylating agent to a nucleophilic centre.

a. Unimolecular mechanism. The separation of the alkyl radical from the rest of the alkylating agent molecule occurs at a rate dependent on the solvent, but once separated R reacts rapidly with any available nucleophilic centre.

$$R - Y \xrightarrow{\text{slow}} R^+ + Y^- \qquad \text{(reversible)}$$

$$R^+ + X^- \xrightarrow{\text{rapid}} R - X$$

b. Bimolecular mechanism. The alkyl radical does not separate completely from the rest of the alkylating agent molecule before being transferred to a nucleophilic centre but is initially loosely combined both to the rest of its molecule and to a nucleophilic centre.

R eventually becomes attached to X as in a unimolecular reaction, but at a rate depending on the concentration and nature of the nucleophilic centres. (For a more detailed discussion of alkylation see Ross, 1962.)

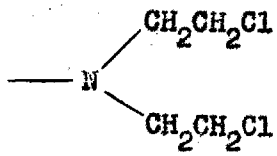
Biological nucleophilic centres. Alkylating agents can react with a number of chemical groups such as organic and inorganic anions, amino groups and sulphide groups. Biological material contains many potential sites for reaction including parts of the nucleic acids and proteins. It is difficult to say which of these chemical reactions cause the important cytotoxic effects of the alkylating agents.

Main Groups of Alkylating Agents

On chemical grounds, the alkylating agents can be divided into four main groups. These are: nitrogen mustards
ethyleneimines
epoxides
methanesulphonates.

A. Nitrogen Mustards

The nitrogen mustards contain a chloroethylamine group in each molecule. The important members of this group in clinical use contain two active groups in each molecule and are known as dichloroethylamines.



If the chloroethylamine group is attached to an aliphatic radical the compound is known as an aliphatic nitrogen mustard and if the chloroethylamine group is attached to an aromatic radical the compound is known as an aromatic nitrogen mustard.

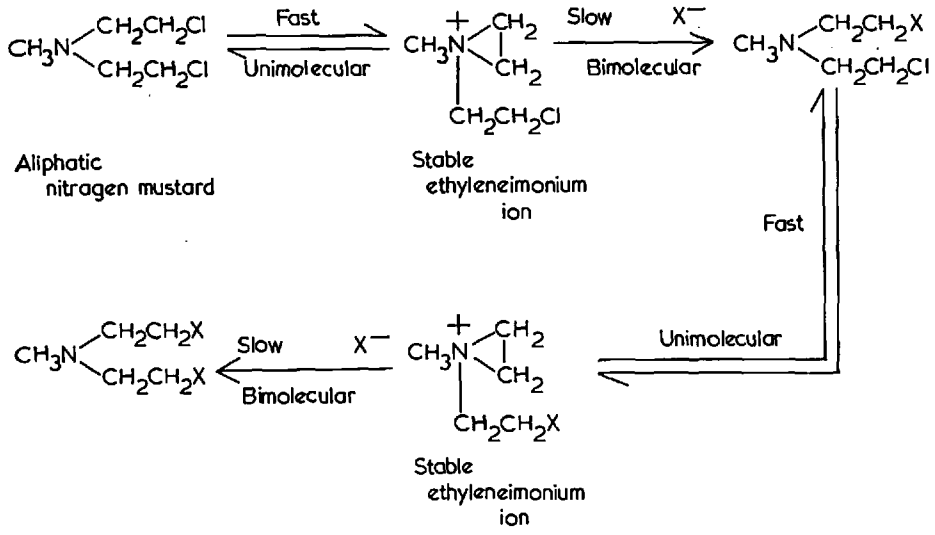


Fig. 1. Reaction of a typical aliphatic nitrogen mustard.

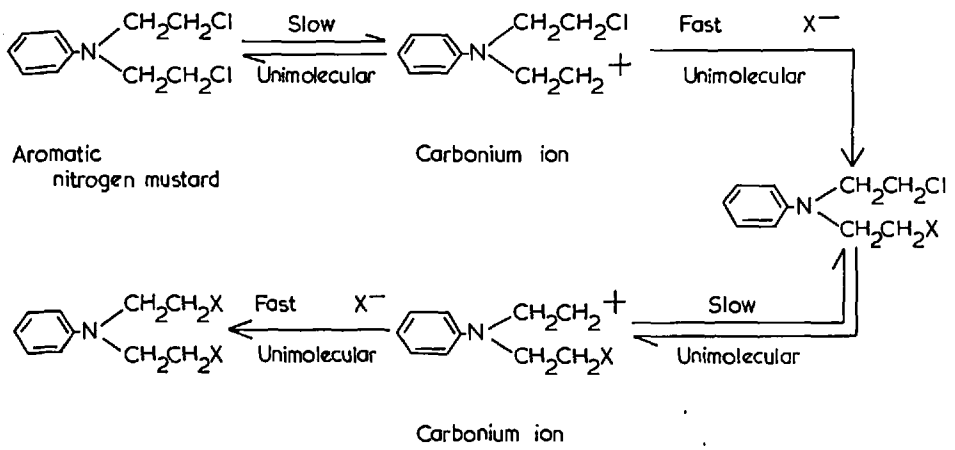
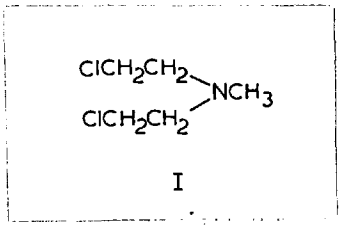


Fig. 2. Reaction of a typical aromatic nitrogen mustard.

Aliphatic nitrogen mustards. Nitrogen mustard (di-2-chloroethylmethylamine) (I) is one of the most commonly used alkylating agents, its hydrochloride being first prepared by Prelog and Stepán (1935). In aqueous solution, a typical aliphatic nitrogen mustard rapidly loses a chloride ion and becomes cyclized to an ethyleneimonium ion (Fig. 1). This three-membered ion is stable and reacts with nucleophilic groups by a bimolecular reaction. Since the dichloroethylamines have two reactive arms in each molecule, each molecule can produce two alkylations.

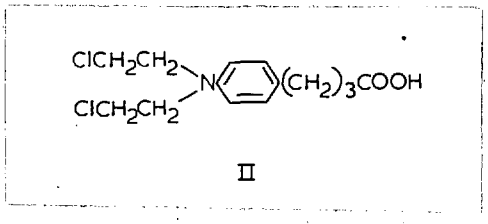


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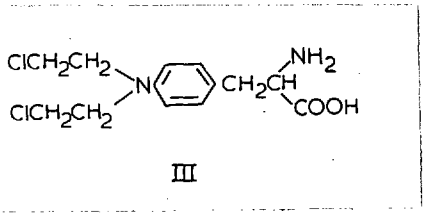
Aromatic nitrogen mustards. The aromatic nitrogen mustards were originally developed by Haddow, Kon and Ross (1948). These compounds have aromatic groups replacing the methyl group of nitrogen mustard. Molecules of drugs in this group eliminate a chloride ion at a slower rate than the aliphatic nitrogen mustards and the carbonium ion formed after this elimination is less stable than the ethyleneimonium ion. Unlike the ethyleneimonium ion formed by the aliphatic nitrogen mustards, the carbonium ion reacts very rapidly with available nucleophilic groups by a unimolecular reaction (Fig. 2). The second arm of dichloroethylamines reacts in the same manner as the first, though the rate of the reaction is influenced by the

nature of the nucleophilic site to which the drug molecule is already attached. The rate of reaction of the aromatic nitrogen mustards is dependent on the rate of formation of the carbonium ions and independent of the concentration of nucleophilic sites. In contrast, because the ethyleneimonium ion is formed rapidly and then reacts by a bimolecular mechanism, the rate of reaction of the aliphatic nitrogen mustards depends on the concentration of nucleophilic sites.

In an attempt to obtain higher activity and specificity, a number of derivatives of the aromatic nitrogen mustards have been prepared. These include the benzene derivatives containing aliphatic carboxylic acid side-chains (Everett, Roberts and Ross, 1953), of which chlorambucil (II) is one of the most successful clinically. Amino acid derivatives have also been prepared, with the hope of these compounds showing some antimetabolite effect. The dl-, l-, and d-isomers of p-(di-2-chloroethylamino)phenylalanine (mer-, mel-, and med-phalan) (III) are examples of this group (Bergel and Stock, 1954). Larionov, Khokhlov, Shkodinskaja, Vasina, Troosheikina and Novikova (1955), in the U.S.S.R., prepared the racemic form independently and called it sarcolysine.



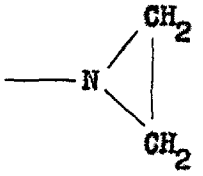
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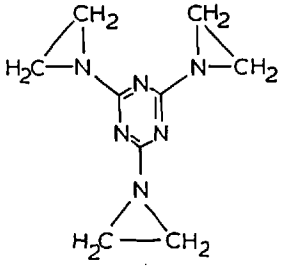
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B. Ethyleneimines

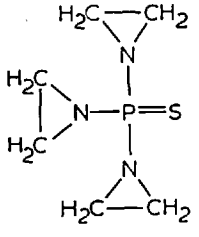
These compounds contain the group



The ethyleneimines are structurally similar to the ethyleneimonium ion formed by the aliphatic nitrogen mustards. The ethyleneimine groups react by a bimolecular reaction, the rate of reaction depending on the nucleophilicity and concentration of the available nucleophilic groups. Thus, the rate of reaction is very slow in a neutral aqueous solution but is faster in the presence of reactive anions and other reactive groups. Most of the active drugs have more than one ethyleneimine group to each molecule. Tretamine (IV) and thioTEPA (V) are triethyleneimines.



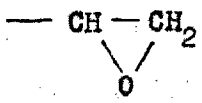
IV



V

C. Epoxides

These compounds contain the group



The epoxides are chemically similar to the ethyleneimines. They contain a three-membered ring which opens in the presence of a nucleophilic group and reacts with it. The rate of reaction is slow in neutral solution but is increased in the presence of electron-rich groups.

D. Methanesulphonates

These compounds developed from Timmis' idea of replacing the reactive Cl^- ion in some nitrogen mustards with the mesyl group,

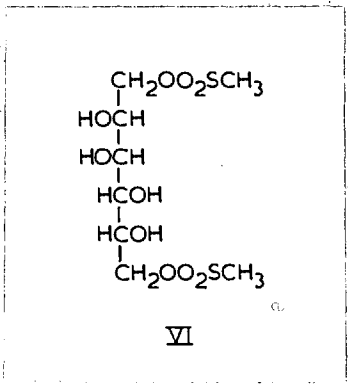
$$- O - SO_2 - CH_3$$

Nitrogen was then eliminated from the molecule altogether and one such compound, 1,4-dimethanesulphonoxybutane (myleran or busulphan), was found to have considerable inhibitory action on the growth of the Walker rat carcinoma 256 (Haddow and Timmis, 1953). When reacting with the nucleophilic groups, the methanesulphonates leave the alkyl radical attached to the nucleophilic group while the methanesulphonoxy part of the molecule is split off.

$$\underline{R}\,CH_2 - O - SO_2 - CH_3 \longrightarrow \underline{R}\,CH_2^+ + CH_3 - SO_2 - O^-$$

The reaction is bimolecular. Many derivatives have been developed,

including mannitol myleran (VI) which has a sugar-group as the carrier of the cytotoxic part of the molecule. This compound, being very soluble in water and an effective inhibitor of various animal tumours when given in relatively high doses (Haddow, Timmis and Brown, 1958), may act by way of epoxide intermediates.



VI

Action of Alkylating Agents in the Cell

The chemical reactions of the main groups of the alkylating agents have been described briefly in the previous section. These reactions give no indication of how alkylation affects the viability and metabolism of the cell. That alkylating agents are extremely active against the living cell has been realised since 1887 when Meyer described the vesicant and local necrotizing actions of pure samples of mustard gas. Many of the early workers thought that mustard gas was a non-specific local poison and that death of the organism was due to secondary infection after extensive necrosis (see review by Philips, 1950). A further idea was that when mustard gas hydrolysed within the cell, it released sufficient H^+ and Cl^- ions to damage sensitive intracellular structures by acidity (Lynch, Smith and Marshall, 1919). An alternative suggestion was that mustard gas might be oxidised *in vivo* to toxic intermediates and that these were responsible for the drug action. One such intermediate, the sulphone, is known to be chemically reactive (Berenblum and Wormall, 1939; Banks, Boursnell, Francis, Hopwood and Wormall, 1946). These early theories have fallen into general disrepute.

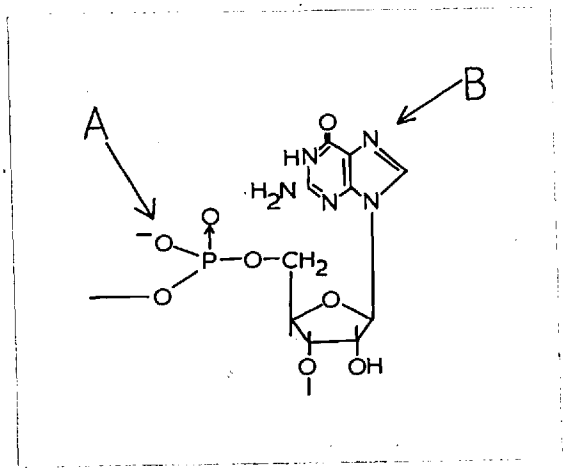
Modern ideas about the important molecular sites of alkylation within the cell stem from the discovery that parts of nucleic acid molecules and protein molecules can act as nucleophilic centres. The effects of alkylating agents on nucleic acids and proteins are discussed in the following sections, together with other possible

modes of damaging the normal cell metabolism.

Drug Action on Nucleic Acids

Since 1940, evidence has built up to suggest that alkylating agents act on the cell nucleus and that deoxyribonucleic acid (DNA) is highly susceptible to damage. There are reports of mustard gas causing irreversible precipitation of nucleoprotein from rabbit skin and calf thymus (Berenblum and Schoental, 1947), reduced fertility and induced mutations in Drosophila (Auerbach and Robson, 1946), chromosome disorganization (Koller, 1958), and inactivation of viruses, particularly those containing DNA (Herriott, 1948). The fact that difunctional alkylating agents (those with two alkylating radicals in each molecule) are generally more cytotoxic than the corresponding monofunctional alkylating agents (those with a single alkylating radical in each molecule) has been related to the ability of the difunctional agents to cross-link the twin strands of the macromolecular structure of DNA (Goldacre, Loveless and Ross, 1949). Moreover, the positions of breaks in chromosomes of dividing cells of Vicia roots treated with various alkylating agents are not random but tend to be concentrated in particular regions, often in the heterochromatin (which has a high nucleic acid content) (Loveless and Revell, 1949).

Although it is generally agreed that DNA function is affected by alkylating agents, there is some dispute about the molecular site of drug action. Some postulate that enzymes essential for DNA



Guanylic acid

Fig. 3. Possible sites of alkylation of the nucleic acid molecule.

A. **Esterification of the phosphate groups of the polynucleotides.**

B. **Reaction with the N-7 atom of the guanine moiety of the polynucleotide.**

Modified from Montgomery (1959).

synthesis are inactivated but that the DNA molecule itself is unaffected. Enzyme inhibition is a feasible idea since mustard gas interferes with the action of certain proteases, phosphokinases and related enzymes (Needham, 1948) and of choline oxidase and acetylcholine esterase (Barron, Bartlett and Miller, 1948). This hypothesis has largely been rejected, however, as the main explanation of the cytotoxicity of alkylating agents, because Philips (1950) has shown that cytotoxic effects can be demonstrated in vivo with mustard concentrations too low to inactivate most enzymes. Considerable experimental evidence has also been produced to show that drug action is not on some subsidiary mechanism but that the DNA molecule is directly alkylated (see review by Wheeler, 1962). The site of alkylation of the DNA molecule is thought by some to be the phosphate groups (Ross, 1958; Stacey, Cobb, Cousens and Alexander, 1958) and by others to be nitrogen atoms of the polynucleotide purines and pyrimidines (Fig. 3). The N-7 atom of the guanine moiety is theoretically the most reactive nucleophilic centre in the nucleic acid molecule (Pullman and Pullman, 1959) and this has been proved experimentally with DNA for a variety of alkylating agents (Lawley and Wallick, 1957; Brookes and Lawley, 1960, 1961). Alkylation of the DNA molecule would presumably interfere with the function of DNA and with cell division and, because of chain breakage, would lead to chromosome disorganization and mutations.

Some of the published experimental results indicate that damage

by alkylating agents is selectively against DNA while others suggest that ribonucleic acid (RNA) is also affected. To some extent, this appears to depend on the drug concentration used, the higher the concentration the more likely is RNA to be involved. Thus, Brewer, Comstock and Aronow (1961) found that RNA synthesis occurred at the normal rate in mouse fibroblasts three days after treatment with 0.1 μg./ml. nitrogen mustard but was depressed three days after treatment with 0.2 μg./ml. Similarly, Pradhan and West (1960) showed that chlorambucil (10 μg./gm./day) inhibited the incorporation of glycine-C^{14} into nucleic acids of a transplantable lymphosarcoma in mice after three days treatment, while a single drug treatment failed to produce inhibition. Examples of the published results are given in Table I. It can also be seen that a relatively high drug concentration in contact with cells for a short time is as effective in inhibiting RNA synthesis as a lower concentration applied for a longer time. As with DNA, Brookes and Lawley (1960) consider that RNA is alkylated at the N-7 atom of the guanine moieties. Alkylation of the RNA molecule would presumably interfere with RNA replication and with protein synthesis.

Drug Action on Proteins

Alkylating agents are highly reactive chemical compounds and only those agents with a minimum chemical reactivity are likely to have anti-tumour properties (Ross, 1958). Although many of the cytotoxic effects produced by alkylating agents on cells appear due

TABLE I. EFFECT OF ALKYLATING AGENTS ON SYNTHESIS OF NUCLEIC ACIDS

Tissue or material	Drug and dose	Reduced synthesis* DNA		RNA
MAMMALS IN VIVO				
	Drysdale, Hopkins, Thomson, Smellie and Davidson (1958)			
Rabbit: Appendix	Mustard gas or nitrogen mustard 1.5 mg./kg. 2-48 hr. before $^{32}PO_4$. 2 hr. incorporation time	+	>	+
	Lawrance and Carter (1950)			
Rabbit: Bone marrow, Spleen, Thymus	Nitrogen mustard 2 mg./kg. Sacrifice 12-48 hr. later. P^{32} 1 hr. before sacrifice	+	>	+
	Goldthwait (1952)			
Rat: Small intestine, Liver	Nitrogen mustard 4 mg./kg. 15 min. after formate-C^{14} or adenine-N^{15}. Sacrifice after 24 hr.	+		-
	Clark and Stoerk (1956)			
Rat: Thymus, Mesenteric node, Spleen	Nitrogen mustard 0.5 mg./kg. x 24 hr. (?). P^{32} 3 hr. before sacrifice	+	=	+
	Pradhan and West (1960)			
Mouse: Lymphosarcoma transplant	Chlorambucil 10 µg./g./day. Glycine-C^{14} $\frac{1}{2}$ - 1 hr. after last dose. Sacrifice 8 hr. later			
	Treated for 1 day	-		-
	Treated for 3 days	+	=	+

* + Reduced synthesis detected
\- No reduced synthesis apparent
= Similar effect on synthesis of DNA and RNA
> Greater inhibitory effect on DNA synthesis than on RNA synthesis

Table I continued

Tissue or material	Drug and dose		Reduced synthesis* DNA		RNA
Mammals in vivo continued					
	Wheeler and Alexander (1964)				
Hamster: Bilaterally grown cyclophosphamide sensitive and resistant tumours	Nitrogen mustard 1 mg./kg./day Tretamine 0.1 mg./kg./day ThioTEPA 4 mg./kg./day Treated for 5 days, then formate-C^{14} or adenine-C^{14}. Sacrifice 2 hr. later				
	Formate-C^{14}:	Sensitive	+	=	+
		Resistant	-		-
	Adenine-C^{14}:	Sensitive	+		-
		Resistant	-		-
MAMMALS IN VITRO					
	Lowrance and Carter (1950)				
Rabbit bone marrow	Nitrogen mustard 100 µg./ml. x 10 min. P^{32} x 1 hr.		+	>	
	Drysdale, Hopkins, Thomson, Smellie and Davidson (1958)				
Ehrlich ascites cells	Nitrogen mustard 50 µg./ml. x 2 hr. $^{32}PO_4$ or formate-C^{14}		+	=	+
MCIM tumour cells	Nitrogen mustard 50 µg./ml. x 2 hr $^{32}PO_4$		+	=	+
	Wheeler and Alexander (1964)				
Minced cyclophosphamide sensitive and resistant tumours previously bilaterally grown in hamsters	Nitrogen mustard 2 µg./ml. added with metabolite. 37° C x 4½ hr.				
	Adenine-C^{14} or hyposanthine-C^{14}:	Sensitive	+		-
		Resistant	-		-
	Formate-C^{14}:	Sensitive	+	>	+
		Resistant	+		-

* + Reduced synthesis detected
\- No reduced synthesis apparent
= Similar effect on synthesis of DNA and RNA
> Greater inhibitory effect on DNA synthesis than on RNA synthesis

Table I continued

Tissue or material	Drug and dose	Reduced synthesis* DNA		RNA
AMPHIBIA				
	Bodenstein and Kondritzer (1948)			
Amblystoma punctatum embryos	Nitrogen mustard 10 µg./ml. x 45 min. Fresh medium for 8 days. Colorimetric estimation of nucleic acids	+		-
BACTERIA				
	Herriott (1951)			
Escherichia coli B.	Mustard gas 70 µg./ml. 37° C x 10 min. Fresh medium for 2 hr. Colorimetric estimation of nucleic acids	+	>	±
	Pardee (1954)			
E. coli B.	Mustard gas 120 µg./ml. 37° C x 10 min. Fresh medium for 40 min. Colorimetric estimation of nucleic acids	+		-
	Harold and Ziperin (1958)			
E. coli B.	Mustard gas or nitrogen mustard x 15 min. Fresh medium for 20 min. Colorimetric estimation of nucleic acids			
	Mustard gas: 85 µg./ml.	+		-
	110 µg./ml.	+	>	+
	Nitrogen mustard: 500 µg./ml.	+		-

* + Reduced synthesis detected
- No reduced synthesis apparent
= Similar effect on synthesis of DNA and RNA
> Greater inhibitory effect on DNA synthesis than on RNA synthesis

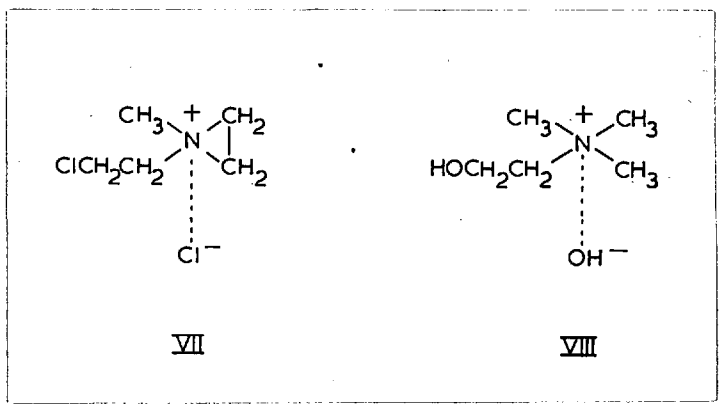
to reaction with nucleic acids (especially DNA), transport of these chemicals to and into cells must bring them into contact with other reactive sites, especially proteins. There are reports of alkylating agents reacting with proteins and a few examples will be given. Butler and Crathorn (1958) found that chlorambucil, melphalan and myleran combine with both the nucleic acid and the protein of rat and calf thymus nucleoprotein although the amount combining with nucleic acid is equal to or greater than the amount combining with protein. Myleran reacted less completely than the nitrogen mustards. Cohn (1957) found that proteins have a higher specific activity from in vivo labelling with C^{14}-phenylalanine mustard than nucleic acids from the same tissues. In spite of this, Brookes and Lawley (1960), reacting S^{35}-mustard gas with tobacco mosaic virus, Bacillus megatherium and Ehrlich ascites tumour cells, found that the presence of protein and other cellular constituents did not prevent alkylation of nucleic acid. The inactivation of enzymes by alkylating agents has been briefly discussed in the previous section. Even though reaction between alkylating agents and proteins can be demonstrated, it does not necessarily follow that such reactions are the prime cause of cytotoxicity.

Cell Cycle Block

In the section on the effect of alkylating agents on nucleic acids, several instances of inhibition of DNA synthesis are cited. In contradistinction to this, other reports show that cell division

can be prevented with concentrations of nitrogen mustard which have no inhibitory effect on DNA synthesis in the same tissues. Ultmann, Hirschberg and Gellhorn (1953) used nitrogen mustard to block cell division in regenerating rat liver and found that the DNA and RNA levels in the treated cells were higher than in the non-treated controls. Similarly, growth of mouse tumours in vivo can be inhibited with doses of myleran, nitrogen mustard, tretamine or triethylene phosphoramides which do not significantly depress the incorporation of P^{32} into DNA of the treated tumour cells (Davidson and Freeman, 1955).

A solution to these apparently contradictory schools of thought about the effect of certain alkylating agents on DNA synthesis is provided by Layde and Baserga (1964) using Ehrlich ascites cells in vivo in mice and by Gelfant (1963). They suggest that nitrogen mustard produces a block in the premitotic resting phase (G2) of the cell cycle (see page 43 for a description of the phases of the cell cycle). Cells in the latter part of the G2 phase at the time of contact with the drug are thought to divide and continue through the following G1 and S phases. These cells are unable to complete a second division because of the block in the G2 phase, but since they have completed the S phase they contain the premitotic level of DNA. Continued DNA synthesis in nitrogen mustard treated cells up to the premitotic level has been shown experimentally by Levis, Spanio and de Nadai (1963) and by Brewer, Comstock and Aronow (1961). Since


CH3
+
CH2
N
ClCH2CH2
CH2
Cl−
VII
CH3
+
CH3
N
HOCH2CH2
CH3
OH−
VIII

RNA synthesis and protein synthesis are thought to be relatively unaffected by the block in the cell cycle, increased dry mass per cell after treatment with nitrogen mustard would not be unexpected (Lee, Richards and Furst, 1961). This hypothesis explains how cell division can be stopped without the immediate inhibition of DNA synthesis and how inhibition of DNA synthesis can occur in experiments of sufficient duration or with concentrations of drug sufficiently high to produce a different cytotoxic effect.

Antimetabolite Action

Because of the similarity between the chemical structures of the ethyleneimonium ion (VII) and choline (VIII) and because choline oxidase is one of the enzymes most sensitive to nitrogen mustard, Barron, Bartlett and Miller (1948) suggested that the ethyleneimonium ion from nitrogen mustard might occupy sites in the cell normally available for choline. Various other attempts have been made to explain the biological effects of the alkylating agents on the grounds of some antimetabolite action. Skipper (1953) proposed that alkylated purines and pyrimidines might be incorporated into DNA and give rise to abnormal products, a feasible idea since the uracil analogue, 5-fluorouracil, and related compounds do show cytotoxic activity. Lorkiewicz and Szybalski (1961) have claimed that tretamine reacts with thymidine mono- or tri- phosphate and that incorporation of these alkylated analogues into DNA is the cause of mutations induced by this drug. This seems unlikely, however, since when

Lawley and Brookes (1963) reacted nucleosides of DNA with excess tretamine under the conditions used by Lorkiewicz and Szybalski, a reaction could be detected with deoxyguanosine only. Timmis (1960) has suggested that alkylated guanine itself might play an anti-metabolite role.

Melphalan. Larionov in 1948 suggested that substituted amino acids that act in the body as antimetabolites might show anti-tumour activity. Melphalan, the l-form of the mustard derivative of the naturally occurring amino acid, phenylalanine, produces complete growth inhibition of Walker rat carcinoma 256 (Bergel and Stock, 1954) and the dl-form produces complete growth inhibition of rat sarcoma 45 and considerable growth inhibition of some other experimental tumours (Larionov, Khokhlov, Shkodinskaja, Vasina, Troosheikina and Novikova, 1955). It is not known, however, whether this anti-tumour activity is due to the phenylalanine mustard acting as an antimetabolite or as an alkylating agent. In favour of the former possibility is the fact that the l-isomer of phenylalanine mustard (melphalan) is more effective than the d- or dl- forms in inhibiting growth of the Walker rat carcinoma 256 in rats and of the Landschütz ascites tumour in mice (Koller and Veronesi, 1956) and is also a more effective inhibitor of uptake of phenylalanine-C^{14} and glycine-C^{14} into Staphylococcus aureus (Crathorn and Hunter, 1957). Moreover, the l-form is about four times as active as the d-form in producing effects on the blood such as a decrease in lymphocyte numbers (Elson,

1954) and is a more active mutagen than the d-form (Fahmy and Fahmy, 1956). The greater activity of the l-form could be due to the natural configuration of the l-form which makes it more suitable for uptake, possibly along pathways involved in protein synthesis. Bergel and Stock (1953) dismiss the possibility that the lesser activity of the d-form is due to its breakdown by d-amino acid oxidase because the rate of attack of these drugs by d-amino acid oxidase is only one-fifth the rate of attack of d-phenylalanine.

In spite of the greater activity of the l-form, other experimental evidence points to lack of antimetabolite activity by melphalan. Cohn (1957) administered p-di-(2-chloroethyl)amino-dl-phenyl(β^{14}C)alanine to rats bearing Walker carcinoma 256 and found that more radioactivity was bound to the protein fractions than to the nucleic acids of the same tissue. Treatment of this labelled protein with reagents expected to break ester and ether bonds removed little activity. Chromatography of hydrolysates of the kidney proteins (the kidney having a greater specific activity than any other tissues) showed no radioactivity in tyrosine or phenylalanine spots, indicating that conversion to one of these amino acids was not taking place. Moreover, since liver protein and plasma albumin could be labelled <u>in vitro</u> with drug (even after hydrolysis of the drug) under conditions in which protein synthesis was unlikely, Cohn concluded that at least part of the binding of the labelled drug to protein did not require the presence of the mustard group and that

a metabolic process was probably not involved.

Cohn's results are difficult to explain, and in the hope of obtaining more information about the incorporation of amino acid analogues into protein, Brookes (1959) synthesised a substance similar to the labelled phenylalanine mustard used by Cohn but lacking reactive mustard groups. This substance, p-di-(2-hydroxy-($^{14}C_2$)ethyl)amino-l-phenylalanine, was allowed to react at 37°C with serum albumin for 24 hours or with rat liver homogenates overnight and the protein was then examined for radioactivity. No activity in the protein fractions could be detected. The compound was also injected into one rat and the specific activity of its tissues was examined. Only protein from the kidney showed a specific activity greater than the background and the value was extremely low even from this tissue. Since the results indicated that p-di-(2-hydroxy($^{14}C_2$)-ethyl)amino-l-phenylalanine was not incorporated into protein, Brookes concluded that the activity found in the tissues by Cohn was due to an alkylation reaction involving the mustard groups of the phenylalanine mustard rather than to incorporation into protein involving peptide links. Brookes assumes, however, that his compound reacts in a manner similar to phenylalanine mustard and his ideas do not explain the reaction of hydrolysed phenylalanine mustard with proteins <u>in vitro</u>. An interesting comment by Brookes was that although the greater effect of the l-form of phenylalanine mustard cannot be ascribed to incorporation into protein it might be connected with ease of transport.

Examples have been given of published reports describing the reaction of the alkylating agents with the nucleic acids and protein and the interference with the metabolism and division of the cell produced by these drugs. It is still not known which of these effects are responsible for the anti-tumour action of some of these drugs.

Use of Alkylating Agents for Cancer Chemotherapy

Alkylating agents were not used for the treatment of neoplastic disease until after 1942 when Goodman and Gilman noted the effects of certain nitrogen mustard derivatives on lymphoid tissue and rapidly dividing cells. A possible use of these compounds against lymphoid neoplasms was suggested and this led to clinical trials. Nitrogen mustard was shown to have a significant activity against Hodgkin's disease and caused limited regressive changes in lymphosarcoma. The clinical results became generally known in 1946 (Gilman and Philips).

Since that time, numerous compounds have been screened for anticancer activity and a few are used clinically, either as an adjunct to surgical excision or for regional therapy. For examples of the clinical applications of the alkylating agents, see Farber, Tooh, Sears and Pinkel (1956), Irvine, Noon and Bastable (1962), and Creech and Krementz (1964).

At the present time, the use of alkylating agents for cancer chemotherapy is hindered by the fact that any anti-tumour activity is usually accompanied by damage to normal tissues. This cell damage,

however, cannot be due in all cases to a general toxic effect on all cells since selectivity of drug action against certain cell types can be demonstrated with some of the alkylating agents.

Selectivity of Drug Action

Since the alkylating agents damage some cell types *in vivo* more than other cell types, some selectivity of drug action must take place. It is usually those cell types with a high mitotic rate that show the greatest damage (lymphoid tissue, bone marrow, intestinal mucosa, germinal cells, skin). It could be argued that these drugs show anti-tumour activity simply because they act on all tissue with a high mitotic rate. If this were true, only those neoplastic cells with a mitotic rate greater than that of normal cells in the same organism would be likely to respond to chemotherapy and the nature of the drug used would be relatively unimportant. Drug action solely against cells with a high mitotic rate cannot be the whole explanation of the anti-tumour activity of some of the alkylating agents because the mitotic rate of tumours rarely exceeds that of normal intestinal mucosa and because, as shown below, some examples of selectivity of drug action against certain cell types disprove this idea.

Connors and Whisson (1966) have demonstrated a correlation between the *in vivo* sensitivities of mouse tumour cell lines to aniline mustard and their glucuronidase contents. It was found that two plasma cell lines with levels of glucuronidase five times

higher than the levels in the mouse liver regressed completely on treatment. Two tumour cell lines with a glucuronidase content equal to that in the liver failed to respond to treatment with this drug. The authors suggest that aniline mustard is converted to the glucuronide in the liver, then broken down by glucuronidase in the tumour to the toxic p-hydroxyaniline derivative. This indicates that the sensitivities of tumours to chemotherapeutic agents can be influenced by factors other than the mitotic rate.

The work of Jackson (1959) on the effect of chemicals on fertility in the rat can be cited as further evidence of the selectivity of drug action against only certain cell types. Myleran is thought to block an early stage in spermatogenesis since fertility is normal until about the eighth week after the drug injection when the rats become sterile. The aliphatic nitrogen mustards appear to interfere with later stages of spermatogenesis since these drugs produce immediate sterility, though potency is later restored. The aromatic nitrogen mustards, on the other hand, have no effect on fertility, even at doses which inhibit tumour growth and suppress bone marrow activity. A single dose of tretamine reduces fertility immediately and causes sterility four weeks after drug administration. Potency is restored by the fifth week, indicating a possible effect on spermatocytes. Repeated doses of tretamine appear to affect all stages of spermatogenesis since this treatment produces prolonged sterility. ThioTEPA produces results similar to those described

for tretamine. Even though most of the cells involved in spermatogenesis are in the same environment, selectivity of drug action for certain cell types can be demonstrated and the nature of the cell types being selectively damaged depends on the drug used.

The work of Elson (1958) on the in vivo effects of myleran and chlorambucil on rat blood cells provides a further example of selectivity of drug action against certain normal cell types. Myleran produces a marked decrease in the number of neutrophils while chlorambucil produces an initial depressant effect on the neutrophils followed by rapid recovery and neutrophilia. In contrast, myleran has no effect on lymphocytes while chlorambucil causes a decrease in lymphocyte number.

Two factors in the effects of alkylating agents on mixed cell types are therefore apparent: (a) there are a few examples of a drug being more cytotoxic to certain cell types than to others; (b) the cell types showing this selective damage depend on the alkylating agent used and not solely on the mitotic rates of these cells. This selectivity of drug action against certain cell types is probably dependent both on the nature of the cells and on the nature of the drug. If the drug is highly reactive, insufficient active drug may reach the target site because of reactions elsewhere in the body. Tissues with a poor blood supply may be less damaged than highly vascular areas. Drug entry into the cell is affected by such factors as the nature of the cell membrane, the lipid and water

solubilities of the drug and the length and nature of the carrier part of the drug molecule. Side-chains such as amino acids and sugars may aid transport and metabolism of the drug in some cells, while the presence of alternative pathways or detoxicating enzymes within the cell would tend to make that cell resistant. The importance of activating enzymes for the sensitivity of tumour cells has already been mentioned. It is probably the interplay of factors such as these which is responsible for the selective action of the alkylating agents.

Whatever the reason for selectivity of drug action against certain cell types within an organism, the fact that selectivity has been demonstrated does indicate that chemotherapy of neoplastic disease without excessive side-effects may be possible. Tumours from each type of human neoplastic disease show a wide spectrum of sensitivities to the drugs in current clinical use. Since these drugs generally produce toxic side-effects in all patients receiving them, and since only those patients with tumour cells sensitive to the drug used will show any beneficial response, a method of determining tumour sensitivity to selected drugs prior to administration would be of great advantage. Drugs to which the cells were resistant could be eliminated and the drugs to which the cells were sensitive could be graded in the order of their ability to inhibit growth of those particular cells. The aim of this investigation was to examine a sensitivity test of this type.

EXPERIMENTAL PLAN

Cell System

Although there is a vast literature on cytotoxic agents, there are few comprehensive comparisons of the actions of different drugs at various concentrations. Published reports cover a wide variety of tissues and experimental conditions (see, for example, Table I on page 22) and it is difficult to find which factors are likely to be important in an *in vitro* sensitivity test for tumours. Do high and low concentrations of a drug affect a cell system in the same way? Is the drug action the same on all cells in the population or is there variation in effect from cell to cell? Is there a single vital metabolic pathway affected by each drug or is the cell damage more general? Are non-dividing cells affected as much as dividing cells? In designing experiments to answer such questions it was felt desirable to exclude as far as possible factors that might vary haphazardly in the tissues examined. For this reason, although the ultimate aim of this work is to investigate the drug sensitivities of tumours, it was decided to avoid the variability inherent in tissues of this sort and to use instead a tissue undergoing rapid proliferation and from which relatively reproducible cell preparations could be made. The tissue chosen was mouse thymus. In the experiments described below, the thymus cells were incubated *in vitro* so that the drug action could be studied directly, without interference from competitive sites elsewhere in the body. The experiments were not intended to provide

a realistic representation of drug action within human neoplastic tissue *in situ*. The object was to determine which factors are likely to be important in a possible *in vitro* sensitivity test for human tumours.

The thymus in the mouse is a creamy-white bilobed organ lying over the anterior ventral surface of the heart. Each of the two main lobes is divided into macroscopic lobules by connective tissue. The inner medullary portion of each lobule is lightly staining and is composed of a meshwork of reticular cells (with pale nuclei) and relatively few lymphocytes which are embedded in this meshwork. The medulla is continuous from one lobule to another. The peripheral area of each lobule, the cortex, is darkly staining and contains numerous tightly packed lymphocytes (with dark nuclei) and relatively few reticular cells. The large, medium and small lymphocytes make up 90 per cent of the weight of the thymus and the large and medium lymphocytes provide 99.9 per cent of its mitotic activity (Metcalf, 1964). The small lymphocytes in the thymus do have a low but definite mitotic rate (Kindred, 1938), unlike the small lymphocytes in other tissues which rarely divide if at all (for example, see Cooper and Alpen, 1959).

The most conspicuous part of the fixed and stained thymus lymphocyte is the nucleus. This is surrounded by a scanty rim of cytoplasm. Palade (1955) reported the presence of an endoplasmic reticulum in all cells examined (mainly from rat tissues) except

the mature erythrocyte. He noticed, however, that the endoplasmic reticulum in lymphocytes from lymph nodes, spleen and thymus was less pronounced than in many other cells. Incorporation of labelled amino acids into lymphocytes has been reported (for example, Cooper and Alpen, 1959; Cooper, 1961) and Trowell (1958) in a review on the lymphocyte suggests that small lymphocytes have an active protein turnover. In view of Palade's findings, it is perhaps safer to refer to uptake of amino acid as such rather than as protein synthesis. Small lymphocytes will also incorporate significant amounts of labelled metabolites into RNA. This has been shown by Lajtha, Oliver and Ellis (1954) for adenine-C^{14} and P^{32}, by Cooper and Alpen (1959) for formate-C^{14}, adenine-C^{14} and cytidine-C^{14} and by Rieke (1960) for uridine-H^{3}.

Classification of the lymphocytes of the thymus presents problems, since there is no sudden transition between the sizes of small, medium and large lymphocytes. Schooley and Berman (1960) suggest that thoracic duct lymphocytes have a unimodal distribution of cell diameter and this finding has been confirmed with thymus cells in the present study. An objection to using size limits to distinguish between large and small lymphocytes in the thymus is that the cell size appears to depend on the fixative used. As a further complication, nitrogen mustard causes an increase in cell size (Levis, Spanio and Nadai, 1963), so that it is difficult to follow the different cell types in drug-treated populations. To overcome some of these difficulties, cells

from the thymus have been called simply thymocytes in the experimental section, and when distinction between potentially-dividing cells (large and medium lymphocytes) and probably non-dividing cells (small lymphocytes) is needed, the actual cell diameters are given.

The action of alkylating agents on DNA replication and cell division has been described above and it was thought that any antimitotic activity should be relatively easy to demonstrate in the rapidly dividing cells (large and medium lymphocytes) of the thymus. These agents can also interfere with synthesis of RNA and protein and can inactivate enzymes and this type of reaction would probably be reflected in the ability of the cells to take up RNA precursors and amino acids, and in this cell system can be studied in cells with a high or a low mitotic activity.

Measurement of Cell Damage

Pappenheimer (1917) made suspensions of rat thymus cells and counted the number of cells stained with trypan blue. Diffusion of dye into the nucleus was considered evidence for cell injury. Vycital, Schrek and Clarke (1953) used the dye exclusion test with safranin to indicate damage to rat thymus cells *in vitro*. Using this method, they found that 50 per cent thymocytes were damaged by 2 μg./ml. nitrogen mustard. The disadvantages of this method are that it tends to be unreliable and it provides no indication of the molecular site of damage.

Comparisons of drug actions have been made on various cell systems,

sometimes in the hope of establishing an in vitro sensitivity test for human tumours. The most popular method seems to be incubation of cell suspensions from trypsin-treated tumour biopsies or culture of tumour fragments in the presence of drug (often at one concentration only), followed by an assessment of cytological damage and cell loss. For examples, see Ambrose, Andrews, Easty, Field and Wylie (1962), Easty and Wylie (1963), Cobb, Walker and Wright (1961), Wright, Cobb, Gumport, Golomb and Safadi (1957). Although damage is measured at the cellular level by microscopic examination, incubation is usually continued for more than 24 hours and some selection of the less sensitive cells may occur.

Other workers make biochemical measurements on the population as a whole. These are probably more accurate than an assessment of cytological change but do not allow for variation from cell to cell or for the presence of dead or non-neoplastic tissue. These methods include measurement of dehydrogenase activity (DiPaolo, 1963), protein content (Eagle and Foley, 1956), oxygen uptake (Onesti and Woodliff, 1963), and uptake of P^{32}-labelled phosphate (Yarnell, Ambrose, Shepley and Tohao, 1964). Scholler and Bittner (1958) and Teller, Palm, Merker, Harris and Woolley (1958) follow the growth of transplanted tumours in drug-treated animals. There are many drawbacks to this method, however, such as the difficulty of obtaining growth of human tumours transplanted into other animals, the unknown influence of the altered environment, the possibility of immunological

attack by the recipient on the transplant and the complication introduced by drug effects on the recipient animal.

A method which eliminates the drawbacks of most other methods is that first proposed by Wolberg and Curreri (1961). The incorporation of uridine-H^3 by tumour slices in the presence of possible anti-tumour drugs is determined autoradiographically. Variation from cell to cell can be measured, allowance can be made for dead or non-malignant cells and following the uptake of a metabolite provides a more exact measure of cell growth than assessment of cytological changes. The method was later extended to include uptake of thymidine-H^3 (Wolberg and Brown, 1962). McDonald, Stroud, Brues and Cole (1963) have used a similar method on established cell lines and on human tumours. Since incubation is continued for about 24 hours in each case, the surviving cells may not represent all the cell types originally present in the tumour. Bickis, Henderson and Quastel (1965) incubate chopped or sliced tumour specimens with drug for a short time (10 min. at $4^{\circ}C$ and 20 min. at $37^{\circ}C$) before the ability of the tissue to incorporate various labelled metabolites in a 2 hour incorporation period is assessed. This method eliminates the drawbacks of long-term incubation of the cells, but since uptake of the labelled metabolites into DNA, RNA and protein is measured by scintillation counting, there is no indication of variation from cell to cell. The ideal <u>in vitro</u> test should include the measurement of drug action (over

a range of concentrations) at the cellular level and should involve short-term incubation only.

Measurement of Thymidine-H^3 Incorporation

From feulgen-photometry (Swift, 1950) and from autoradiographic studies (Howard and Pelc, 1951), DNA synthesis is known to occur during part only of interphase. The cell cycle can be conveniently subdivided into the phases suggested by Howard and Pelc (1953). Mitosis is followed by a period (G1) in which no DNA synthesis occurs and which may continue until the death of the cell. If cell division is to take place, a DNA synthetic phase (S) follows. Between the S phase and mitosis is a phase (G2) during which no further DNA synthesis takes place, the cells containing twice the normal DNA content. In general, all cells which are to divide must first duplicate their DNA content. Therefore, following uptake of marked metabolites which can be utilized for DNA synthesis provides a convenient method of showing which cells have the capacity to divide. Cells which have synthesised DNA as far as the premitotic level will not necessarily divide, but those which have not completed the S phase are unlikely to do so.

Thymidine is a popular metabolite for this purpose since it is a specific DNA precursor (Reichard and Estborn, 1951; Friedkin, Tilson and Roberts, 1956; Friedkin and Wood, 1956; Amano, Messier and Leblond, 1959). The label from labelled thymidine can be recovered from the cell nucleus only, in the thymine of DNA. Purines

and pyrimidines from RNA show no significant level of labelling. This has been shown for a variety of tissues and under a variety of experimental conditions. Tritium (H^3) provides a convenient marker for the thymidine, enabling high resolution autoradiography because of the short range of the β-particles (Taylor, Woods and Hughes, 1957). Since treatment in absolute ethanol or methanol has been found to remove all precursor pool tritium (Zajicek, Bernstein, Rosin and Gross, 1963), it is reasonable to assume that cells containing tritium after this treatment have been synthesising DNA at some time during their exposure to thymidine-H^3.

Thymidine needs to be used with caution. Excess thymidine has been used to produce synchrony and blockage of the cell cycle (Petersen and Anderson, 1964). There are also reports of it prolonging the G1 phase (Van't Hof, 1965), inhibiting the incorporation of uridine into nucleolar and cytoplasmic RNA (Kasten, Strasser and Turner, 1965) and even stimulating cell division (Greulich, Cameron and Thrasher, 1961). The quantity of thymidine-H^3 used was kept to a minimum for this reason and also to minimise any radiation damage from the tritium. Since the thymidine-H^3 is taken into the cell nucleus, radiation damage is a definite hazard and there are reports of thymidine-H^3 causing growth inhibition (Painter, Drew and Hughes, 1958), change in ploidy classes (Post and Hoffman, 1961), and chromosome and mitotic aberrations (Wimber, 1959; Natarajan, 1961). All these experiments, however, involved a relatively high concentration of thymidine-H^3 and

incubation for at least several hours.

A further complication in the use of thymidine is that some tissues contain an enzyme, thymidine phosphorylase, which degrades thymidine to thymine (Friedkin and Roberts, 1954). In Ehrlich ascites cells, this degradation has been shown to take place at a faster rate than incorporation of thymidine into DNA and since thymine is not incorporated into DNA this results in a lowering of the effective concentration of thymidine (Zajicek et al., 1963).

Although labelling with thymidine-H^3 is a good qualitative indication of DNA synthesis, it is not strictly quantitative. A fairly constant correlation between the number of grains in the photographic emulsion and the number of tritium atoms in the underlying tissue section has been found by Kisieleski, Baserga and Vaupotic (1961).

In conclusion, thymidine-H^3 can be a useful tool in the assessment of cell metabolism but needs to be used with caution.

EXPERIMENTAL WORK

Material and Methods

Mice

The mice were purchased from Glaxo Laboratories Limited and were used at 9 to 33 g. Female A2G mice were used for the majority of the experiments. Male or female C57Bl, (A2G x C57Bl)F_1 hybrids or Pirbright albinos were used, however, for some of the initial experiments. Until used, the mice were kept 12 to a cage in a room maintained at 70°F and were fed on 41B mouse diet and water *ad libitum*. Whenever possible, the thymocytes used in each individual experiment came from one mouse. This was not possible in experiments involving large numbers of cells.

Nutrient Medium

The thymocytes were incubated in Hanks' balanced salt solution (Glaxo Laboratories Limited) enriched with 12.5 per cent serum which had been previously heated at 56°C for ½-hour. Except for a few of the initial experiments, in which horse serum (Burroughs Wellcome) was used, pooled mouse serum was added. Penicillin and streptomycin were also added to give a final concentration of approximately 100 and 75 units respectively per ml. It was realised that the presence of these antibiotics could have had some effect on the metabolism of the cells but they were included since their presence is likely to be necessary for human neoplastic tissue which is often infected.

Labelled Metabolites

The labelled metabolites were purchased from the Radiochemical Centre, Amersham, and were diluted in physiological saline to the appropriate concentrations. The batch numbers and specific activities of the labelled metabolites used were as follows:

Labelled metabolite	Batch number	Molecular weight	Specific activity
Thymidine-H^3	19	242	4.32c / mM
	30		8.8 c / mM
Uridine-H^3	5	244	1.57c / mM
l-Tyrosine-H^3	4	181	670mc / mM
l-Leucine-H^3	2	131	215mc / mM
	3		200mc / mM

The amino acids used were generally-labelled with tritium. The tritiated thymidine used (TRA 61), being prepared from tritiated thymine by enzymatic synthesis, had all the tritium activity in the thymine part of the molecule. The tritiated uridine used in these experiments (TRA 27) was prepared by an exchange reaction and, although most of the tritium was in the uracil part of the molecule, some was present in the ribose part. The labelled metabolites were added to 1 ml. aliquots of cell suspension in a volume of 0.1 ml.

Cytotoxic Agents

Nitrogen mustard, di-2-chloroethylmethylamine hydrochloride. "Mustine hydrochloride" (Boots). Molecular weight 192.5.

Chlorambucil, p-(di-2-chloroethylamino)phenylbutyric acid. Kindly supplied by Dr D. A. G. Galton, Chester Beatty Research Institute, London. Molecular weight 304.2.

Melphalan, l-p-(di-2-chloroethylamino)phenylalanine. "Alkeran" (Burroughs Wellcome). Molecular weight 305.2.

Mannitol myleran, 1,6-(dimethanesulphonyl)-1,6-dideoxymannitol. Kindly supplied by Dr G. M. Timmis, Chester Beatty Research Institute, London. Molecular weight 338.

Tretamine, triethylenemelamine (I.C.I.). Molecular weight 204.2.

ThioTEPA, triethylenethiophosphortriamide (Lederle). Molecular weight 189.2.

Methotrexate, 4-amino-N^{10}-methylpteroylglutamic acid (Lederle). Molecular weight 454.5.

Diluents

Sterile, pyrogen-free distilled water was used to dissolve and dilute nitrogen mustard, mannitol myleran, tretamine and thioTEPA. Chlorambucil and melphalan, being insoluble in water, were dissolved in absolute ethanol and acid alcohol respectively and diluted in propylene glycol diluent (all these reagents were supplied by the manufacturer). All tubes in the chlorambucil and melphalan series were adjusted to contain the same alcohol (or acid alcohol) and propylene glycol concentrations. Sodium bicarbonate solution (5%) was added in a volume of 0.1 ml. to each 1 ml. aliquot of cell suspension in

the melphalan series to neutralise the acid. Methotrexate was dissolved in sodium bicarbonate solution, then diluted in water, and each tube in this series was adjusted to contain the same concentration of sodium bicarbonate. Each drug (or the appropriate diluent in the control tubes) was added to 1 ml. aliquots of cell suspension in a volume of 0.1 ml.

Thymus Cell Suspensions

The mouse was weighed and killed with anaesthetic ether. The thymus was aseptically removed into a petri dish containing nutrient medium and teased with fine scissors and forceps. The crude cell suspension was then filtered through gauze to remove cell clumps. The cell concentration was measured in a Neubauer haemocytometer in a sample of suspension which had been diluted in 1 per cent acetic acid. The cell concentration in the remaining cell suspension was adjusted if necessary by the addition of nutrient medium. For most experiments, a concentration of 3×10^6 cells/ml. nutrient medium was used.

Incubation Procedure

The cells were studied in 1 ml. aliquots in 16 x 150 mm. or 16 x 120 mm. pyrex test tubes fitted with Oxoid aluminium caps. Incubation at 37°C in a hot-air incubator was performed with the tubes in the vertical position and without gassing or shaking. Cells were incubated with a labelled metabolite for various times. Labelling was stopped by centrifuging the cells at 650 g. and 4°C for 5 to 10

minutes and washing the cells in two changes (occasionally a single change) of 5 per cent bovine plasma albumin (Armour) in saline. Thymidine-H^3 labelling was stopped in a few of the initial experiments by the addition of excess unlabelled thymidine (100 µg./ml. cell suspension) followed by immediate cooling of the suspension to 4°C, before the usual washing in bovine plasma albumin. The supernate from the final wash was discarded and smears were made from the cell pellet on to grease-free slides which had been previously treated with a solution of 0.5 per cent gelatin and 0.1 per cent chrome alum and dried. The smears were rapidly air-dried. Cells incubated with thymidine-H^3 or uridine-H^3 were fixed in methanol overnight. Cells incubated with either of the labelled amino acids were fixed in Bouin's solution for 10 min. and rinsed briefly in 70 per cent and 50 per cent ethanol. After fixation, the smears were allowed to dry in air.

Autoradiographic Procedure

Kodak AR 10 stripping film was left standing at 4°C over a saturated solution of calcium nitrate before use to produce an appropriate degree of humidity in the autoradiographic film (Gude, 1957). The film on each plate was divided into 8 sections as recommended by the manufacturer and the sections were floated on distilled water at 24° to 25°C for 3 min. with the gelatin backing uppermost. Each slide was then taken from distilled water and coated with a section of stripping film with the photographically

sensitive surface next to the cells. The slides were allowed to dry at room temperature overnight before being transferred to a light-tight box at 4°C. In most experiments, the film was exposed to the tritium for 7 to 14 days, depending on whether the smears were being examined for the number of grains over labelled cells (the shorter exposure time) or for the percentage cells labelled (the longer exposure time). The film was then developed for 5 min. in freshly made D19B developer (Analar or Kodak chemicals), rinsed in distilled water and fixed for 12 min. in Johnson's Fix-sol (diluted 1 : 10 in distilled water). The slides were rinsed in distilled water for the next hour, the water being changed every 10 min., then the slides were allowed to dry in air at room temperature. All solutions used for the processing were cooled to 17°C before use and all stages involving use of stripping film were performed in a dark-room (illuminated with a Kodak no.2 safelight), until fixation was complete.

Cell smears were routinely stained, after processing, in 1 part Leishmann to 3 parts Giemsa made up to 100 parts M/100 phosphate buffer at pH 6.8 (slightly modified from Lajtha, 1954). The stain was filtered and cooled to 13° to 15°C before use. The smears were stained for 10 to 15 min., then differentiated in the phosphate buffer for another 10 to 15 min. The slides were dried in air, mounted, and given random code numbers before examination under oil-immersion objective. At least 30 labelled cells were examined on each smear to give a mean value for the number of grains over labelled cells. When

the percentage of cells labelled on a smear was determined, the cells were examined in batches of 100 cells and, in most experiments, a total of at least 1,000 cells was examined on each smear.

Measurement of Cell Diameter

The cells were fixed and stained in the usual manner and examined with an oil-immersion objective. An eye-piece graticule was calibrated against a stage micrometer viewed under the same magnification. Cell diameter was measured in one direction only since the outline of most thymocytes was approximately circular; any thymocytes with an elongated form were ignored. Although not strictly accurate, this method allowed large numbers of cells to be examined in a reasonable time and did allow differentiation between obviously large and obviously small cells.

Estimation of Alkylating Radicals

The method used was that described by Boyland, Staunton and Williams (1961). One ml. of the sample containing the alkylating agent was mixed with 3 ml. of isotonic sucrose (0.25 M). Three ml. of this mixture was transferred to a second test tube containing 0.5 ml. pyridine solution (5% w/v p-nitro benzyl pyridine in methyl-ethylketone) and 0.1 ml. acetate buffer (0.6 M, pH 4.5). This tube was then heated in a waterbath at 80°C for 30 min. (chlorambucil estimation) or 20 min. (nitrogen mustard estimation). The contents of the tube were cooled in ice-water and centrifuged at approximately 260 g. for 5 min. to spin down the protein precipitate. Two ml. of

a triethylamine and acetone mixture (50% v/v) were added to 2 ml. of the supernate and 2 to 3 min. after the addition of this last reagent the absorbance of light by the sample at 565 μ was measured on a Perkin-Elmer 137 UV spectrophotometer, using a glass cuvette. The samples were read against air.

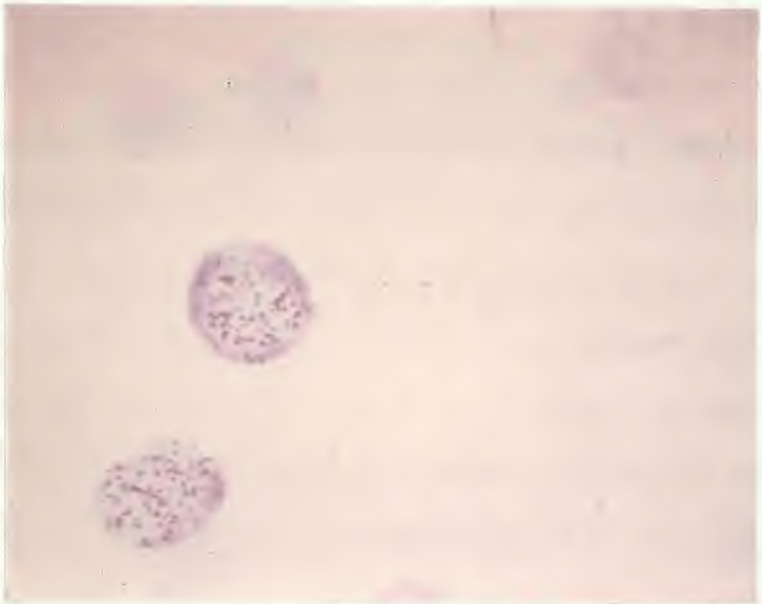


Fig. 4. Mouse thymocytes labelled with thymidine-H^3 *in vitro*. Approximate magnification X 1200.

RESULTS

1. Uptake of Labelled Metabolites by Untreated Cells

Thymidine-H^3 Uptake in the Absence of Cytotoxic Agents

To determine whether autoradiographic detection of incorporated thymidine-H^3 is a feasible estimate of cell metabolism in this cell system, a series of experiments was performed in the absence of any cytotoxic agent. Labelling was apparent in the cell nucleus only (Fig. 4) and since fixation for 3 min. in absolute ethanol or methanol is said to remove all precursor pool tritium from cells incubated with thymidine-H^3 (Zajicek et al., 1963), the presence of silver grains over cells was assumed to indicate thymidine-H^3 incorporation into DNA. Increasing the exposure time of the stripping film to the β-particles from the tritium increased the number of grains over each labelled cell but had little effect on the percentage of cells labelled. For this reason, the percentage of cells labelled was determined from smears exposed for 14 days, after which time the labelled cells could be distinguished from the unlabelled cells very easily. This exposure time, however, resulted in some cells being covered by grains too numerous to count with accuracy and the mean number of grains over each labelled cell was, therefore, determined from smears exposed for 7 days only. Cells covered by less than 4 silver grains were recorded as unlabelled to avoid confusion with background labelling. (On a typical autoradiograph exposed for 14 days, the level of background labelling was calculated to be less than one silver grain on average over each cell.)

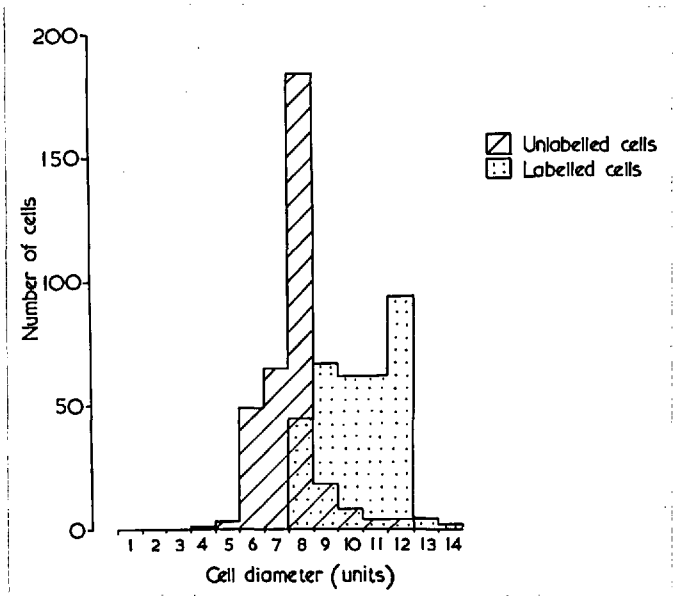


Fig. 5. Correlation between cell size and uptake of thymidine-H^3 by mouse thymocytes <u>in vitro</u>.
1 unit = 1.16 μ.

Correlation between Cell Size and Ability to Incorporate Thymidine-H^3

It was noticed in the pilot experiments that very few small cells became labelled with thymidine-H^3. To check this impression, autoradiographs were prepared of mouse thymocytes which had been incubated for $\frac{1}{2}$-hr. in nutrient medium (1 ml.) with 0.1 ml. saline and 0.1 μc thymidine-H^3, and the diameters of an equal number (336) of labelled and unlabelled cells were measured against an eye-piece graticule. The cell diameters ranged from 4 eye-piece units to 14 units (4.6 μ to 16.2 μ) and all the labelled cells were found to have a diameter of 8 or more units (9.25 μ or more) (Fig. 5; Appendix Table 1). In this investigation, no labelled cell was found with a diameter of less than 8 units (9.25 μ) although on other occasions a few cells with a diameter of 8.6 μ (or rarely 7.1 μ) have been found labelled with thymidine-H^3. It is possible either that the small sized cells in the methanol-fixed material corresponded to small thymocytes not synthesising DNA, or that these cells were of the same type as the labelled cells and were synthesising DNA but failed to show labelling with thymidine-H^3 because, through insufficient flattening in some parts of the cell smear, their nuclei were too far from the stripping film for any emitted β-particles to enter the photographic film. The latter idea can be rejected because unlabelled cells were randomly intermingled with the labelled cells throughout the smear. It is therefore concluded that, in methanol-fixed material, cells with a diameter less than 9 μ correspond to small, rarely-

TABLE II. LACK OF EFFECT ON THYMIDINE-H^3 UPTAKE OF MIXING THYMOCYTES FROM DIFFERENT MOUSE STRAINS (an incorporation period of 35 min. was allowed for the thymidine-H^3)

Incubation time (hr.) before addition of thymidine-H^3	Source of thymocytes (mouse strain) (12×10^6 cells / ml.)		
	Pirbright	(A2G x C57Bl) F_1	Pirbright plus (A2G x C57Bl) F_1
	Grains per labelled cell (± standard deviation)		
0	27 ± 14.6	33 ± 14.9	26 ± 15.6
$1\frac{1}{2}$	32 ± 11.4	32 ± 11.1	35 ± 14.1
	Percentage of cells labelled (± standard deviation)		
0	15.2 ± 4.3	10.0 ± 2.7	13.4 ± 3.4
$1\frac{1}{2}$	17.6 ± 2.5	16.9 ± 3.4	14.8 ± 2.8

dividing thymocytes and those with a diameter greater than 9 μ to medium and large thymocytes. Cells with a 9 μ diameter may belong to either group. Unlabelled large and medium cells are those not in the S phase during the incubation period with thymidine-H^3. The mean grain count per labelled cell due to uptake of thymidine-H^3 was similar for cells of all sizes though large cells tended to have slightly higher grain counts. No strong correlation was found between the cell radius and the mean grain count per labelled cell (Fig. 12 on page 73, and Appendix Table 2).

Lack of Effect on Thymidine-H^3 Uptake of Mixing Thymocytes from Different Mouse Strains

Since the same thymidine-H^3 uptake was obtained with a mixed cell suspension made from part of a thymus from a Pirbright albino mouse and part of a thymus from an (A2G x C57Bl)F_1 hybrid as with the pure cell populations from the rest of either of the two thymuses alone, it was assumed that mixing thymuses from mice of the same strain would have no marked effect on thymidine-H^3 uptake over the short incubation times used. (Table II).

Cell Concentration

Cell concentration was found to have a marked effect on the uptake of thymidine-H^3. The mean number of grains over labelled cells and the percentage of cells labelled was found to be inversely proportional to the cell concentration (Fig. 6, Appendix Table 3). Thus, with 0.8×10^6 cells/ml., 14.6 ± 2.5 per cent cells became

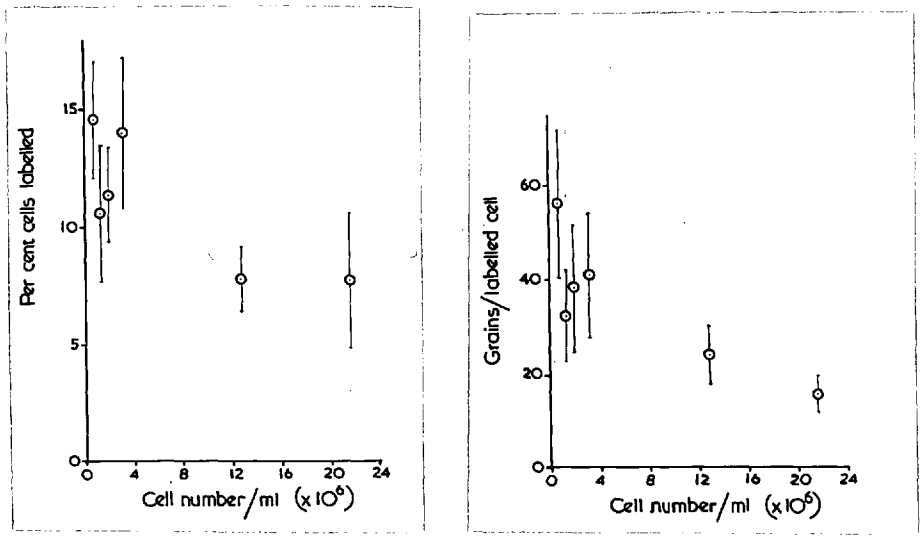


Fig. 6. The effect of cell concentration on thymidine-H^3 uptake by mouse thymocytes <u>in vitro</u>.

labelled in an incubation period of 35 min. and showed a mean grain count of 56.2 ± 15.6 grains/cell, whereas with 21.6 x 10^6 cells/ml. incubated with the same quantity of thymidine-H^3 for the same time, 7.7 ± 2.9 per cent cells became labelled with a mean grain count of 15.6 ± 3.9 grains/cell. If it can be assumed that each grain over a cell nucleus is equivalent to one arbitrary unit of incorporated thymidine-H^3, then the 0.8 x 10^6 cells had incorporated 656 x 10^6 arbitrary units of thymidine-H^3 and the 21.6 x 10^6 cells had incorporated 2594 x 10^6 arbitrary units. These results may reflect competition for the thymidine-H^3 itself, or they may be due to the fact that a dense population of cells grows at a slower rate than a less dense population of cells because of competition for growth factors in the medium or because of an accumulation of waste products from the cells. These results indicate that it is essential that the cell concentration should be constant in comparisons of thymidine-H^3 incorporation into different cell aliquots. Cell concentration is difficult to standardize in tissue slices but it is relatively easy to control in a cell suspension.

Thymidine Phosphorylase

Decreased thymidine-H^3 uptake by a large number of cells could be due to degradation of the thymidine-H^3 by a relatively high concentration of thymidine phosphorylase (see Introduction, page 45). Less enzyme might be produced by a low concentration of cells and therefore less thymidine-H^3 would be degraded. To determine whether

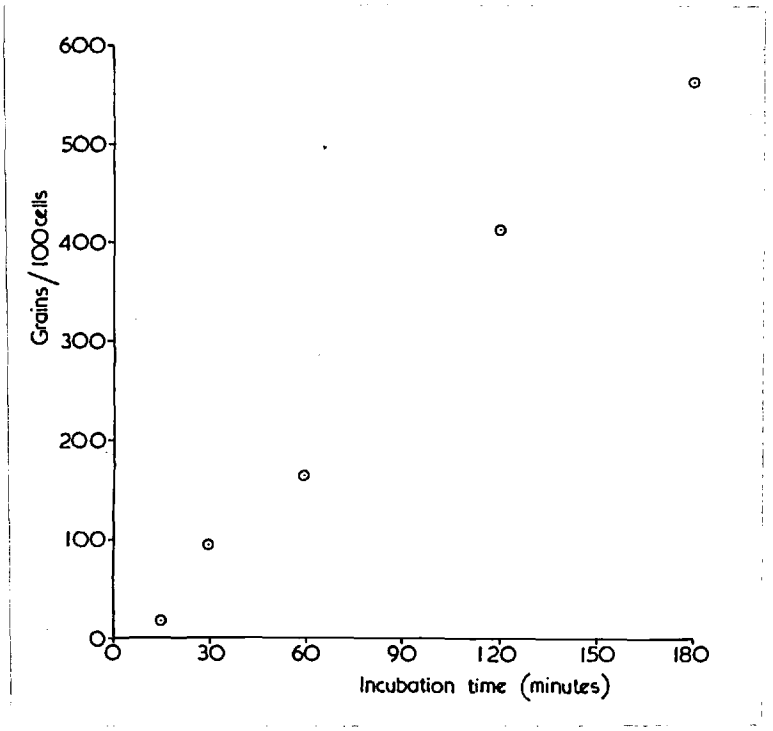


Fig. 7. Rate of thymidine-H^3 uptake by mouse thymocytes <u>in vitro</u>.

thymidine phosphorylase activity was likely to be a complicating factor in this cell system, thymidine-H^3 uptake was examined for up to 3 hr. Zajicek et al. (1963) found that total thymidine-H^3 uptake by ascites tumour cells fell off after the first 20 min. continuous incubation with thymidine-H^3 (1.0×10^{-4} µM thymidine-$H^3/10^8$ cells) but that uptake was increased by the addition of more than 0.35 µM unlabelled thymine/10^8 cells. With mouse thymocytes incubated in vitro with thymidine-H^3, the grain count/100 cells (equivalent to Zajicek's total thymidine-H^3 uptake measured by scintillation counting) showed an approximately linear increase with time throughout the 3 hr. (Fig. 7, Appendix Table 4). Moreover, addition of 3.97 µM unlabelled thymine/6.2×10^6 cells did not increase labelling with thymidine-H^3 (2.32×10^{-5} µM thymidine-H^3/ 6.2×10^6 cells) but tended to decrease it.

These results with mouse thymocytes also conflict with results reported by Cooper and Milton (1964) for uptake of thymidine-H^3 by human leucocytes, the thymidine-H^3 being degraded to thymine and dihydrothymine after 1 hr. at 37°C. To determine whether the differences in findings were due to experimental procedures, mouse thymocytes were prepared in the usual manner and their ability to incorporate thymidine was examined by Dr E. H. Cooper. A cell suspension (8.6×10^6 cells/ml.) was prepared from the thymus of a 21 g. female (A2G x C57Bl)F_1 hybrid mouse and incubated in a water-bath at 37°C with thymidine-C^{14}. Samples were taken at intervals

TABLE III. LACK OF DEGRADATION OF THYMIDINE-C^{14} BY MOUSE THYMOCYTES IN VITRO

Time after addition of thymidine-C^{14} (min.)	Percentage of counts recovered as thymidine (duplicate readings)	
0	58.7	57.6
10	57.7	56.3
20	54.7	56.1
30	52.7	57.5
60	50.8	52.9

TABLE IV. RATE OF INCORPORATION OF THYMIDINE-H^{3} INTO DNA BY MOUSE THYMOCYTES IN VITRO

Time (min.)	Specific activity of extracted DNA (scintillation counts)		
	Duplicate counts		Average
0	29.9	103	66.5
15	830	688	759
45	2230	2300	2265
90	4030	4330	4180

from duplicate tubes, centrifuged, and the supernatant fluid separated into components chromatographically. The radioactivity in the various components was counted on a scintillation counter. The results (Table III) show that mouse thymocytes cause very little degradation of thymidine in the medium.

Since thymidine phosphorylase activity has not been detected, the lower thymidine-H^3 uptake by large numbers of cells than by small numbers is unlikely to be due to the presence of this enzyme.

In a further experiment, the specific activity of DNA from mouse thymocytes incubated with thymidine-H^3 was followed. The cell suspension was prepared in nutrient medium (with horse serum) and contained pooled thymocytes (3.64×10^6 cells/ml.) from the thymuses of two male (A2G x C57Bl)F_1 hybrid mice. Duplicate cell samples were incubated at 37°C with thymidine-H^3 and the specific activity of DNA (estimated by Burton's method (1956) at various intervals after the addition of thymidine-H^3) was determined by scintillation counting. The specific activity of the DNA showed a linear increase with time (Table IV), confirming the autoradiographic findings for mouse thymocytes. Similar results showing lack of degradation of thymidine by mouse thymocytes have since been published (Hale, Cooper and Milton, 1965).

It can be concluded that mouse thymocytes provide a convenient cell system in which to study the effects of cytotoxic agents on thymidine-H^3 incorporation. There is negligable breakdown of

labelled thymidine by these cells and thymidine-H^3 is incorporated into DNA at an approximately linear rate for at least 3 hr.

Duration of Experiments

Lymphocytes in general are known to be difficult to maintain in a suspension culture *in vitro* for more than a few hours (Trowell, 1965) and it was important to know how long the thymocytes could be kept *in vitro* before metabolic functions began to fail. A mouse thymus was made into a cell suspension in the usual manner and 1 ml. aliquots were incubated at 37°C for 0 to 3 hr. Thymidine-H^3(0.1 µc) was then added and incubation continued for a further hr. Labelling was stopped by the addition of excess unlabelled thymidine (100 µg.) and the cells were cooled to 4°C before being washed in 5 per cent albumin in saline. It was found that the same quantity of thymidine-H^3 was taken up in the hr. incorporation period whether the cells were examined immediately after excision of the thymus or after an initial 3 hr. incubation at 37°C (Fig. 8, Appendix Table 5). In a further experiment in which the lag period was extended up to 25 hr., it was again found that the labelling ability of the cells was the same after a 3 hr. lag as immediately after excision. Uptake fell off after a lag of 5½ hr. and became negligable after a lag of 25 hr. (Fig. 8, Appendix Table 5). Because of these results, it was thought advisable to limit drug tests on freshly prepared mouse thymocytes to a maximum duration of 3 hr.

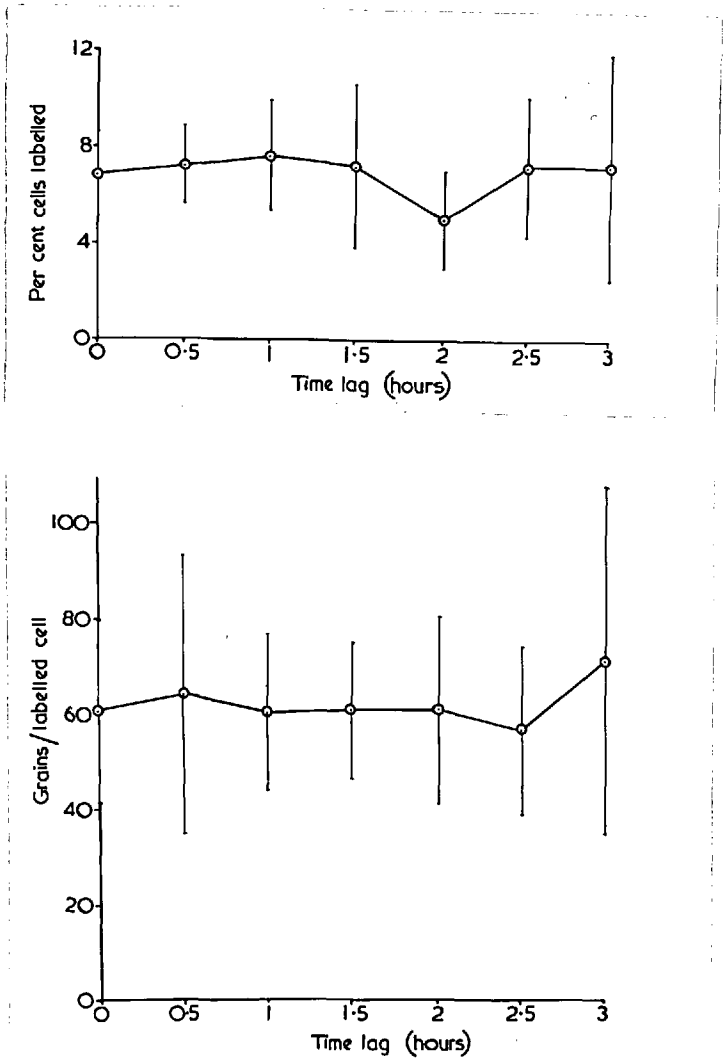
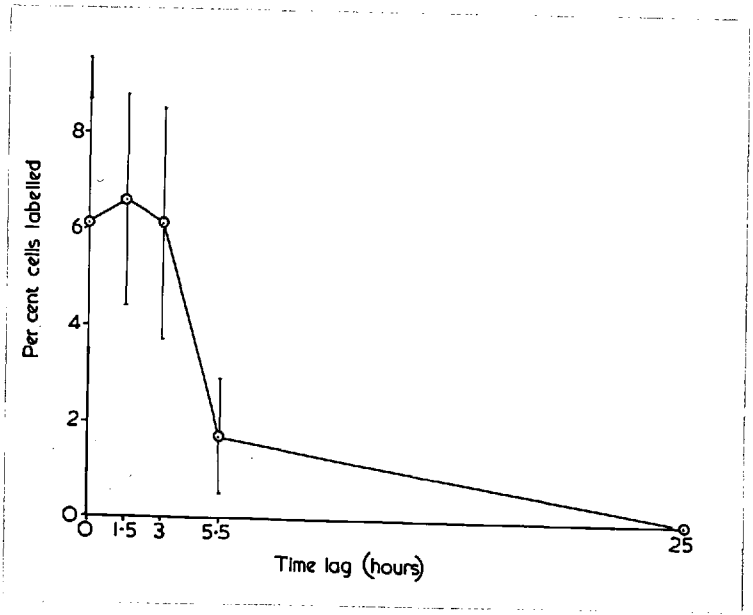


Fig. 8. Experiment 1

Fig. 8. The effect on labelling of a time lag at 37° C between the preparation of a thymocyte suspension and inoubation with thymidine-H^3: two experiments.



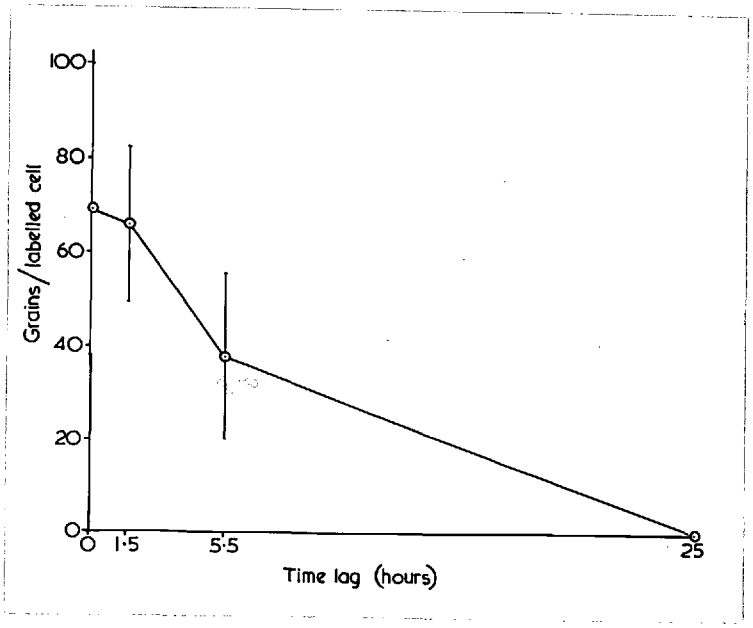


Fig. 8. Experiment 2

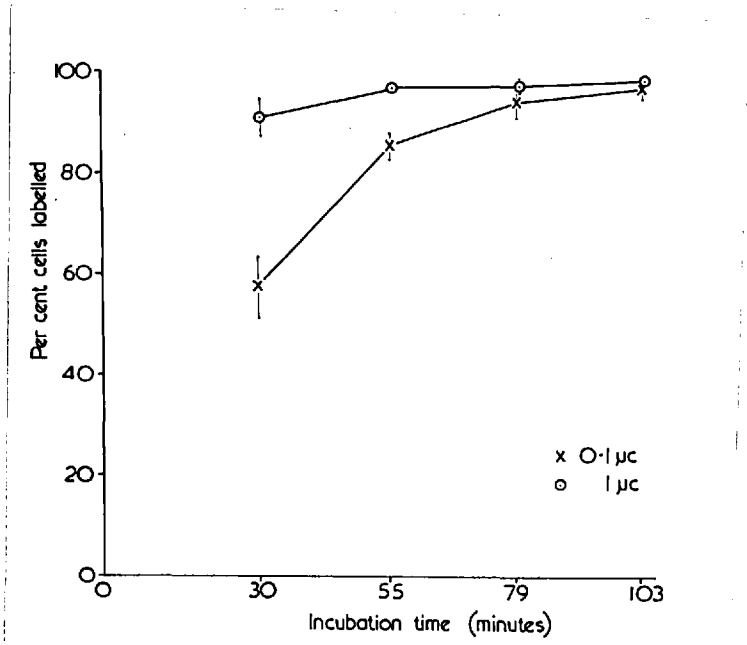


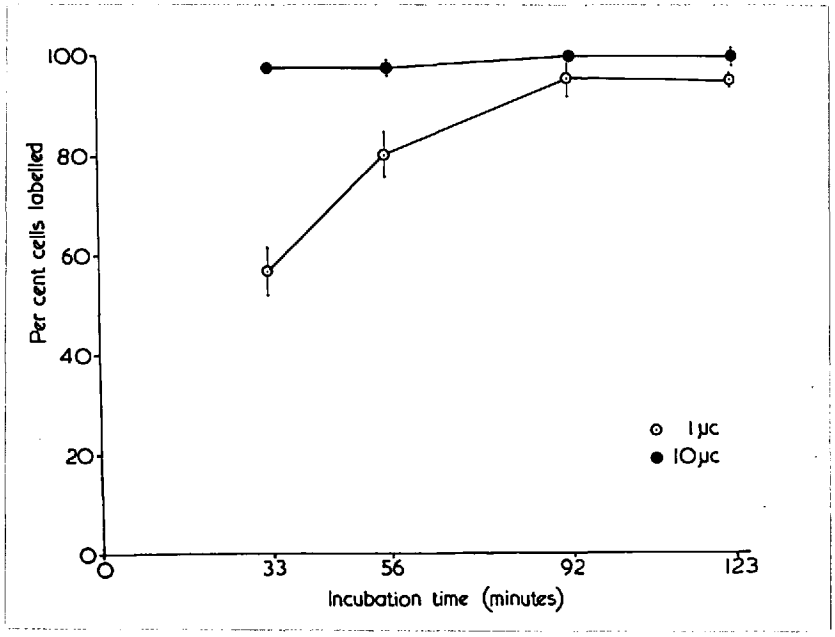
Fig. 9. The uptake of tyrosine-H^3 by mouse thymocytes <u>in vitro</u>.

Uptake of Tyrosine-H^3 and Leucine-H^3 in the Absence of Cytotoxic Agents

Tyrosine-H^3 and leucine-H^3 uptake by thymocytes was examined in tissue taken respectively from a 29.8 g. and a 28.9 g. female A2G mouse. The cell concentration in the tyrosine-H^3 study was 10.2×10^6 cells/ml. and in the leucine-H^3 study was 8.8×10^6 cells/ml. The label was added to 1 ml. aliquots of cell suspension in a volume of 0.1 ml. and labelling was stopped at intervals from $\frac{1}{2}$ to 2 hr. Ninety-nine per cent of the cells became labelled after 103 min. incubation with 1 μc tyrosine-H^3 (Fig. 9, Appendix Table 6) and after 92 min. incubation with 10 μc leucine-H^3 (Fig. 10 and 11, Appendix Table 7), showing that both potentially dividing and rarely dividing cells are capable of incorporating these amino acids under these conditions.

Correlation between Cell Size and Uptake of Tyrosine-H^3 and Leucine-H^3

Cooper and Alpen (1959) noted that autoradiographs of rat thoracic lymphocytes which had been incubated *in vitro* with phenylalanine-C^{14} showed more grains over large and medium lymphocytes than over small lymphocytes. They suggest that this is probably due to the relative protoplasmic masses of the cells. Similarly, large and medium cells from human lymph nodes incorporated a greater quantity of leucine-H^3 *in vitro* (twice the uptake per square micron) than small cells (Cooper, 1961). A similar effect was noticed in



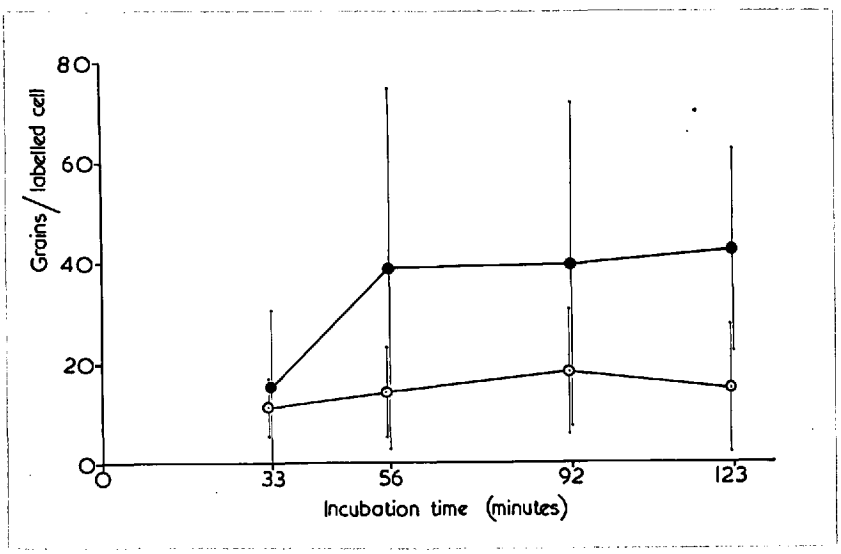


Fig. 10. The uptake of leucine-H^3 by mouse thymocytes <u>in vitro</u>.

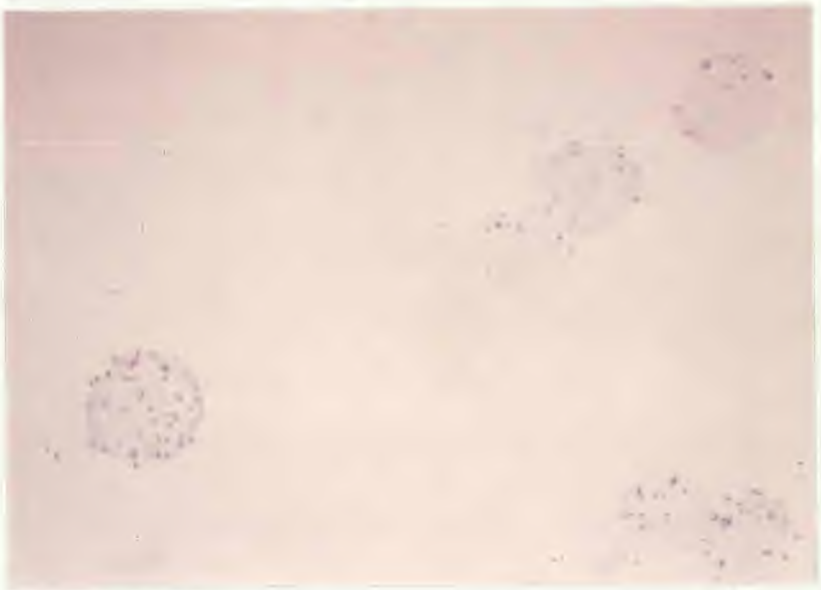


Fig. 11. Mouse thymocytes labelled with leucine-H^3 *in vitro*. Approximate magnification X 1200.

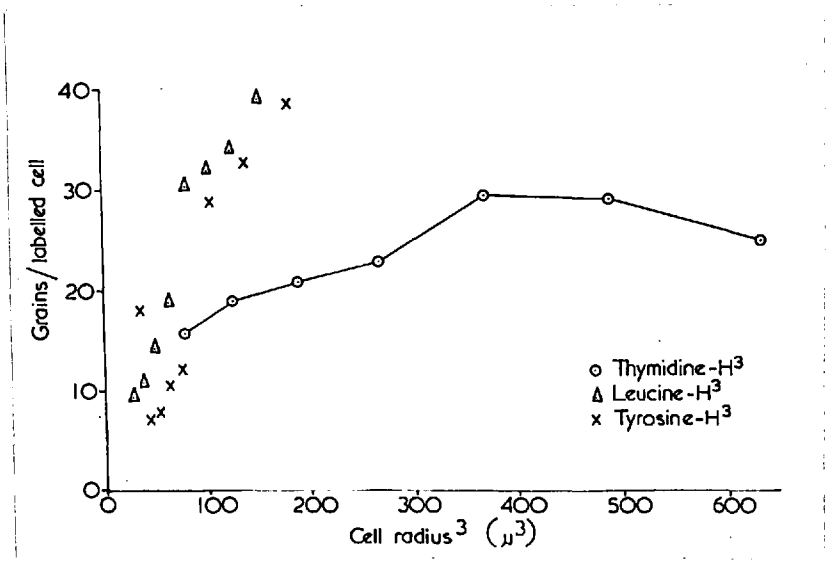


Fig. 12. Correlation between thymocyte size and the grain number over cells labelled <u>in vitro</u> with thymidine-H^3, tyrosine-H^3 or leucine-H^3.

mouse thymocytes and, in an attempt to obtain quantitative information, diameters of cells incubated with 10 μc/ml. leucine-H^3 for 33 min. were measured and the grains per cell counted. The results are given in Fig. 12 and Appendix Table 8. A similar experiment was performed using 1 μc tyrosine-H^3/1 ml. aliquot of cell suspension and 35 min. incubation time (Fig. 12 and Appendix Table 9). In both cases, on plotting the mean grain count per labelled cell against the cube of the cell radius, an approximately straight line was obtained which passed through the origin.

In a 100 per cent efficient autoradiograph, the number of silver grains in the photographic emulsion would be proportional to the amount of radioactive material beneath the grains. The average distance travelled by a β-particle emitted from tritium is 1 μ. Particles emitted more than 1 μ away from the stripping film have a small chance of striking it and contributing towards the production of a silver grain. The efficiency of an autoradiograph in detecting material containing tritium is thus limited by the thickness of the material. The thymocytes when smeared are likely to have been more than 1 μ thick (Pelc, 1966). If the large cells were thicker in smears than the small cells, then the efficiency of the autoradiograph over the large cells would have been relatively low and the grain count would have been an underestimate of the radioactive content of the large cells. The efficiency of counting a large number of grains over a cell is also lower than the efficiency of counting a small

number because of the lower resolution in the former case. It is likely that the large cells would have been covered by an even greater number of grains if a fully efficient autoradiograph could have been produced. The correlation between the grain count and the cell radius is apparent even within the size ranges of the small thymocytes as one group and within the size ranges of the medium and large thymocytes as a second group. There is, however, some indication of a break in the curve at a cell diameter of about 8 μ which may reflect a difference between these two groups. If the autoradiographs had been fully efficient, and assuming that the large cells are thicker when smeared than the small cells, then the difference between the two groups would have been even more apparent. From these considerations, it is suggested that the potentially dividing cells (the medium and large thymocytes) may incorporate amino acids to a greater extent than the rarely dividing cells (the small thymocytes).

With thymidine-H^3 incorporation, no strong correlation is found between the cube of the cell radius and the number of grains over labelled cells; uptake is obviously influenced by other factors (such as the length of the S phase passed while in contact with thymidine-H^3). Fig. 12 also indicates the effect of fixatives on the cell diameters in smears, the cells incubated with thymidine-H^3 being fixed in methanol and the cells incubated with the labelled amino acids being fixed in Bouin's fluid. Other smears have been

examined to check these figures, and methanol-fixed cells consistently have larger diameters and a wider size range than cells fixed in Bouin's fluid.

2. Effects of Cytotoxic Agents

Effect of Nitrogen Mustard on Thymidine-H^3 Uptake by Mouse Thymocytes

Pilot experiments were performed with nitrogen mustard to determine the drug concentrations and incubation times needed to produce an effect on the incorporation of thymidine-H^3 by mouse thymocytes. Typical results are given. The thymuses of two 17 g. Pirbright albino mice were made into a cell suspension containing 3.6×10^6 cells/ml. nutrient medium containing unheated horse serum (Burroughs Wellcome). The nitrogen mustard was dissolved in saline immediately before use and various concentrations were added in a volume of 0.1 ml. to 1 ml. aliquots of cell suspension. Saline (0.1 ml.) was added to two control tubes and all the tubes were gently agitated to ensure mixing. Thymidine-H^3 (0.1 ml.) was added to one set of tubes containing each nitrogen mustard concentration immediately after the addition of drug (no pretreatment), to a second set of tubes containing each nitrogen mustard concentration after the cells had been incubated with drug at 37°C for ½-hr (½-hr. pretreatment), to a third set of tubes after the cells had been incubated with drug for 1 hr. (1 hr. pretreatment) and to a fourth set of tubes after 1½ hr. incubation with drug (1½ hr. pretreatment).

Incubation schedule

Th-H^3 = addition of thymidine-H^3
HN2 = addition of nitrogen mustard

No pretreatment

HN2
Th-H^3 ½ hr.

½ hr. pretreatment

HN2 ½ hr. Th-H^3 ½ hr.

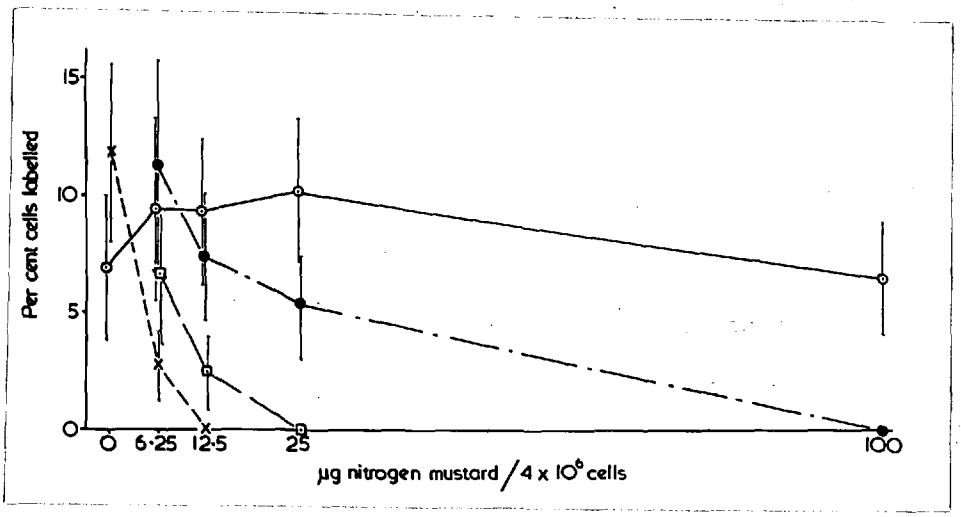
1 hr. pretreatment

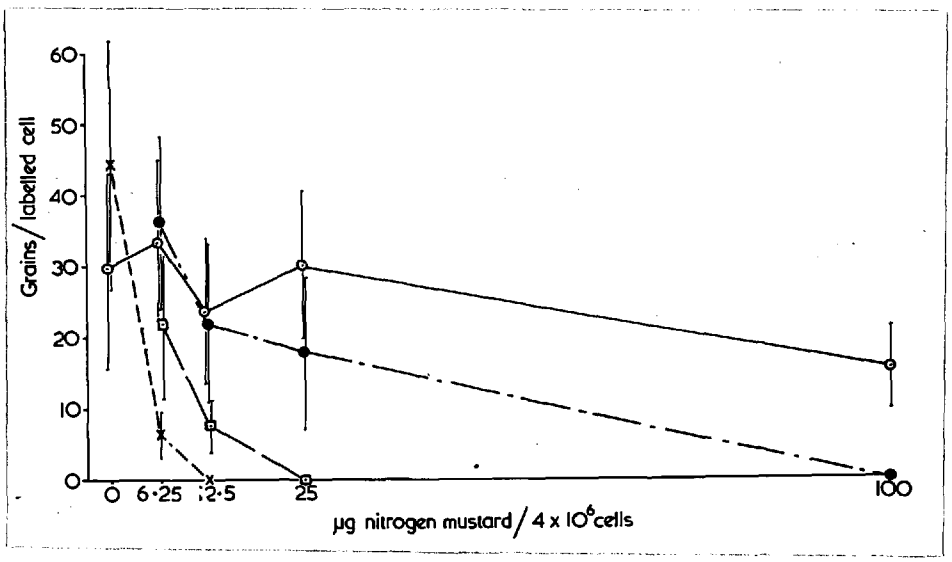
HN2 1 hr. Th-H^3 ½ hr.

1½ hr. pretreatment

HN2 1½ hr. Th-H^3 ½ hr.

Thymidine-H^3 was added to one saline control tube immediately after the addition of the saline and to a second after the cells had been incubated with the saline for 1½ hr. (1½ hr. pretreatment). The final concentration of thymidine-H^3 in each tube was 0.1 μc/ 3.6 x 10^6 cells. In each case, 30 min. incorporation time at 37°C was allowed for the thymidine-H^3, after which time the cells were washed twice in bovine plasma albumin in saline and smeared on slides. The time between removal of the thymuses from the freshly killed mice and the addition of the cells to the tubes was about 15 min. and there was a gap of about 10 min. between the beginning of the preparation of the nitrogen mustard solutions and the addition of thymidine-H^3 to the first batch of tubes. Autoradiographic procedure was as previously described with the exception that the percentage of cells labelled was determined from smears exposed to the stripping film for 7 days





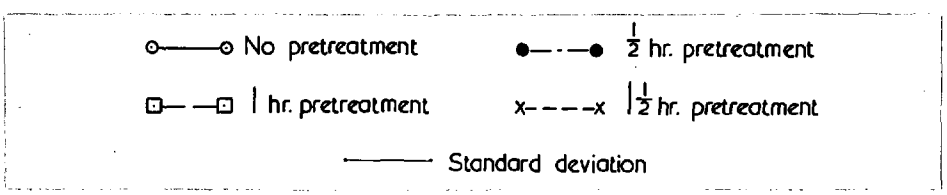


Fig. 13. The effect of nitrogen mustard (diluted in saline) on thymidine-H^3 uptake by mouse thymocytes <u>in vitro</u>.

only. The results are given in Fig. 13 and Appendix Table 10 which show that the thymidine-H^3 uptake (percentage cells labelled and grains/labelled cell) depends not only on the drug concentration but also on the pretreatment time. The longer the pretreatment time with one drug concentration, the fewer the number of cells labelled and the fewer the number of grains over each labelled cell.

It was thought desirable to determine whether this pretreatment effect was peculiar to the action of nitrogen mustard on the uptake of thymidine-H^3 by this cell system or whether it would occur with other labelled metabolites and with other cytotoxic agents.

Comparative Effects of Several Alkylating Agents on Thymidine-H^3 Uptake

The effects of melphalan, chlorambucil, tretamine, nitrogen mustard, thioTEPA and mannitol myleran on uptake of thymidine-H^3 by mouse thymocytes were examined, with a range of drug concentrations and three pretreatment times. Uniform experimental conditions were used to allow a comparison of the drug effects.

Female A2G mice, approximately 4 weeks old and weighing 9.3 to 15.6 g. were used as the source of thymocytes. The serum used to enrich the Hanks' balanced salt solution was taken from a single batch of pooled and inactivated A2G mouse serum, and sufficient thymidine-H^3 solution was made up in saline for the whole series. A separate mouse was used for each drug examined and in each case the cell suspension was adjusted to a cell concentration of 3×10^6 cells/ml. nutrient

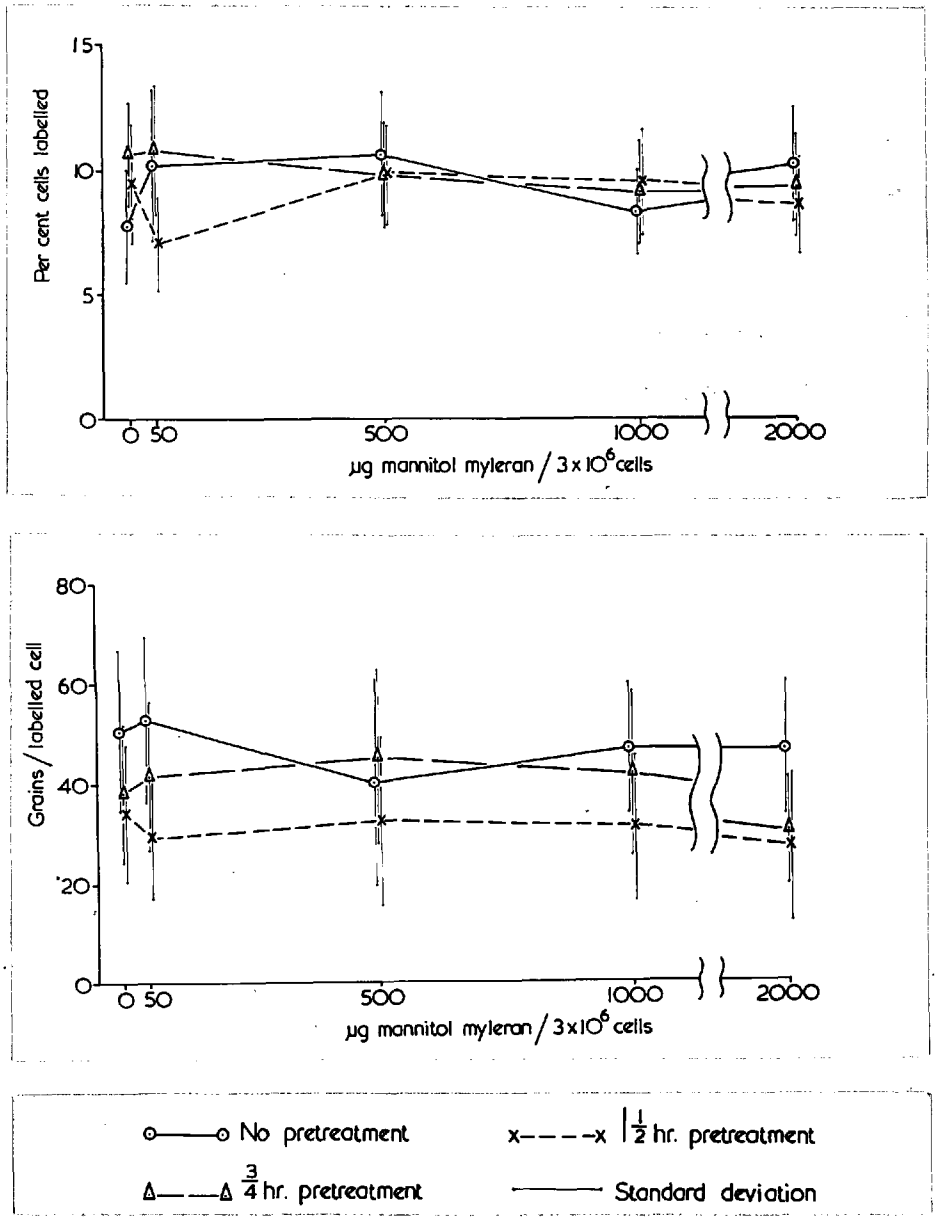


Fig. 14. The effect of mannitol myleran on thymidine-H^3 uptake by mouse thymocytes <u>in vitro</u>.

medium. The drugs were diluted and added to the cell suspensions as described in Material and Methods (page 48). Thymidine-H^3 (0.1μc in 0.1 ml.) was added to one group of tubes immediately after addition of the drug (no pretreatment), to a second group of tubes after ¾ hr. pretreatment of the cells with drug at 37°C, and to a third group of tubes after 1½ hr. pretreatment. The appropriate diluent controls were included in each group.

Incubation schedule

Th-H^3 = addition of thymidine-H^3
Drug = addition of drug (or diluent)

No pretreatment

Drug
Th-H^3 35 min.

¾ hr. pretreatment

Drug 45 min. Th-H^3 35 min.

1½ hr. pretreatment

Drug 90 min. Th-H^3 35 min.

Thirty-five min. incorporation time was allowed for the thymidine-H^3 in each case, after which time the cells were washed twice in bovine plasma albumin solution, smeared on glass slides and fixed overnight in methanol. Autoradiographs were prepared as described above. Sixty labelled cells were examined on each smear exposed for 7 days to yield a mean value for the number of grains over labelled cells and a total of 3,000 cells was examined on each slide exposed for 14 days to yield a mean value for the percentage of cells labelled.

With the exception of mannitol myleran (Fig. 14 and Appendix

TABLE V. DRUG CONCENTRATIONS (mM) CAUSING "COMPLETE INHIBITION" AND 50 PER CENT REDUCTION OF THYMIDINE UPTAKE BY MOUSE THYMOCYTES

Drug and pretreatment time (hr.)	Complete inhibition		50 per cent reduction	
	Based on results from grains per labelled cell	Based on results from percentage of cells labelled	In grains per labelled cell	In percentage of cells labelled
Nitrogen mustard				
0	>2.16	>2.16	0.13	>2.16
¼	>0.026 <0.043	>0.026 <0.043	0.015	0.013
1½	0.022	0.022	0.0087	0.0087
Tretamine				
0	>4.08	>4.08	3.77	>4.08
¼	>4.49 <5.51	>4.08	3.12	>4.08
1½	>2.65 <4.08	>2.45 <4.08	2.14	2.77
Mannitol myleran				
0	>4.93	>4.93	>4.93	>4.93
¼	>4.93	>4.93	>4.93	>4.93
1½	>4.93	>4.93	>4.93	>4.93
ThioTEPA				
0	>5.28 <8.81	>4.84 <8.81	5.88	5.64
¼	>4.62 <5.28	>5.06 <8.81	4.12	4.87
1½	>2.64 <4.40	>2.64 <4.40	3.57	3.41
Chlorambucil				
0	>1.23 <1.37	>1.15 <1.37	1.12	1.15
¼	>1.23 <1.37	>1.19 <1.37	0.68	1.19
1½	>1.23 <1.37	>1.15 <1.37	1.13	1.07
Melphalan				
0	>3.41	>3.41	>3.41	>3.41
¼	>1.70 <3.41	>1.55 <3.41	1.51	2.11
1½	>0.66 <1.31	>0.41 <1.31	0.83	0.73

Table 11), all the alkylating agents examined reduced the percentage of cells labelled with thymidine-H^3 and the mean number of grains per labelled cell below the control values (Fig. 15-19, Appendix Tables 12-16). There was, however, a wide difference in the concentrations of the various drugs needed to produce a comparable effect. Thus, with 1½ hr. pretreatment, 5 μg. nitrogen mustard (0.02 mM) had the same effect on thymidine-H^3 uptake as 1 mg. thioTEPA (4.40 mM). Mannitol myleran, up to 2 mg. / 3×10^6 cells (4.93 mM), had no effect on uptake of thymidine-H^3, even after 1½ hr. pretreatment. As with nitrogen mustard diluted in saline, a pretreatment effect was noticed with four of the drugs, though the extent of the effect varied from one drug to another. The pretreatment effect was pronounced with nitrogen mustard and melphalan, moderate with tretamine and thioTEPA and absent with chlorambucil.

To obtain an estimate of the comparable effectiveness of these drugs in reducing thymidine-H^3 uptake by mouse thymocytes, the probable molar concentrations causing 50 per cent and 100 per cent reduction (from the control values) in the mean grain count per labelled cell and in the percentage of cells labelled were judged from Fig. 14-19. Since these curves have relatively few points, the drug concentrations producing "complete inhibition" of detectable uptake of thymidine-H^3 were difficult to assess. The values given are the lowest and highest drug concentrations likely to have this effect. The concentrations are given in Table V. From these figures,

TABLE VI. ORDER OF DRUG EFFECTIVENESS JUDGED IN TERMS OF THE MOLAR CONCENTRATION NEEDED TO PRODUCE THE DESIRED EFFECT

End-point	Pretreatment time (hr.)	Based on results for percentage of cells labelled	Based on results for grains per labelled cell
Complete inhibition of detectable thymidine uptake	0	Chl > others	Chl > others
	$\frac{3}{4}$	HN2 > Chl > PAM > TEM, ThioT	HN2 > Chl > PAM > TEM, ThioT
	$1\frac{1}{2}$	HN2 > PAM ≥ Chl > TEM, ThioT	HN2 > PAM ≥ Chl > TEM, ThioT
50 per cent reduction from control value	0	Chl > others	HN2 > Chl > others
	$\frac{3}{4}$	HN2 > Chl > PAM > TEM, ThioT	HN2 > Chl > PAM > TEM > ThioT
	$1\frac{1}{2}$	HN2 > PAM > Chl > TEM > ThioT	HN2 > PAM > Chl > TEM > ThioT

Chl = Chlorambucil
HN2 = Nitrogen mustard
PAM = Melphalan
TEM = Tretamine
ThioT = ThioTEPA

the drugs were placed in the order of their ability to interfere with thymidine-H^3 uptake by mouse thymocytes (Table VI). The drugs that appeared to be the most effective depended on the time schedule and on the criterion of inhibition used. Using complete inhibition of detectable uptake as the end-point, chlorambucil was the most effective drug without pretreatment, with $\frac{3}{4}$ hr. pretreatment nitrogen mustard was the most effective with chlorambucil taking second place, and with $1\frac{1}{2}$ hr. pretreatment nitrogen mustard was again the most effective but with melphalan taking second place. In general, the same order was obtained for each pretreatment time whether percentage cells labelled or grains per labelled cell was examined and whether 100 per cent or 50 per cent reduction from the control value was taken as the end-point. The exception to this was the order obtained without pretreatment and with 50 per cent reduction of the main grain count per labelled cell taken as the end-point. Chlorambucil was the most effective drug in reducing the proportion of cells labelled but nitrogen mustard was the most effective in reducing the grain count per labelled cell.

The importance of these results is that if the drugs behaved in a similar manner on human neoplastic tissue in an <u>in vitro</u> sensitivity test, the recorded sensitivity of the tissue would depend on which time schedule was used and, to some extent, on whether grains per labelled cell or percentage cells labelled was examined. The pretreatment effect was, therefore, examined further.

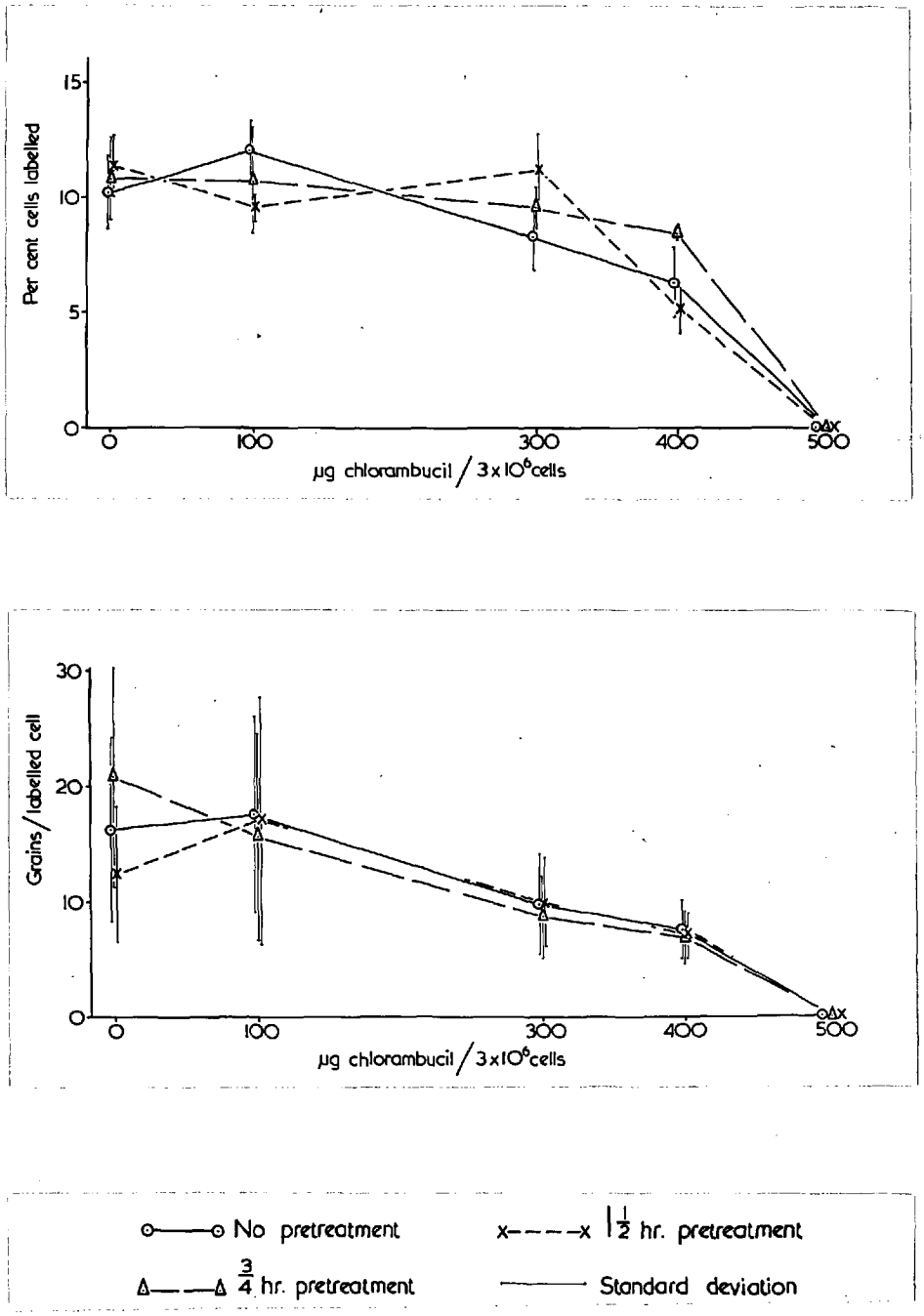


Fig. 15. The effect of chlorambucil on thymidine-H^3 uptake by mouse thymocytes <u>in vitro</u>.

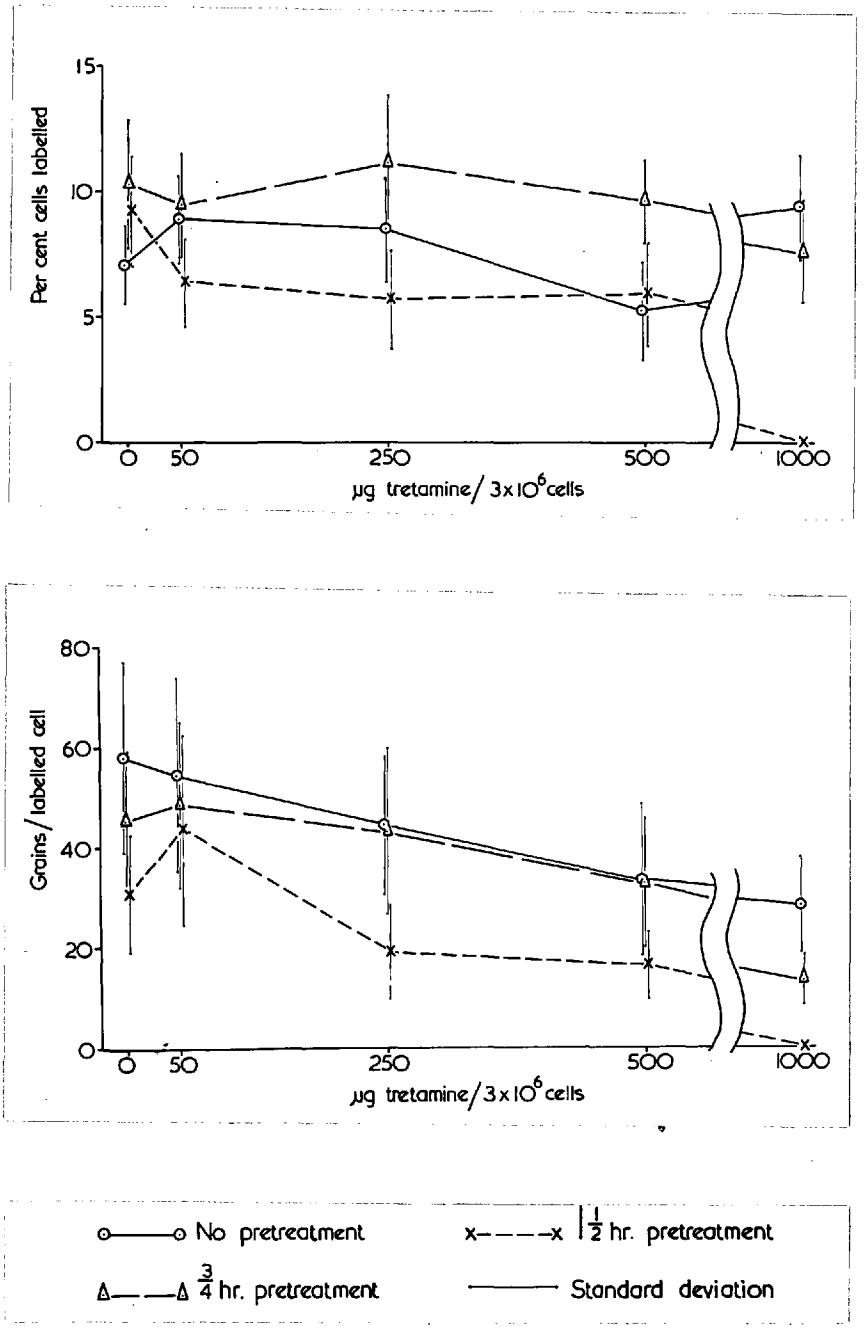


Fig. 16. The effect of tretamine on thymidine-H^3 uptake by mouse thymocytes <u>in vitro</u>.

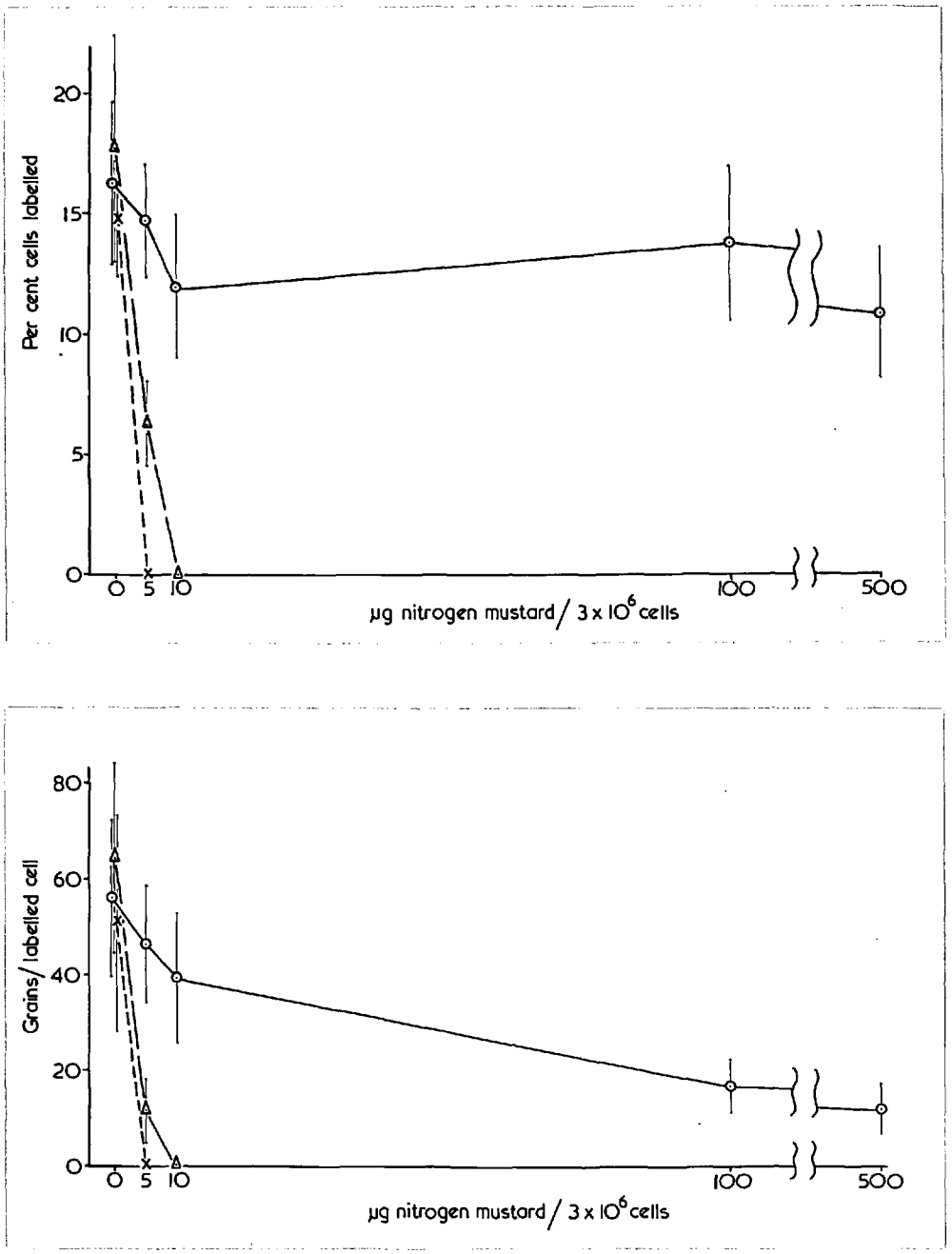
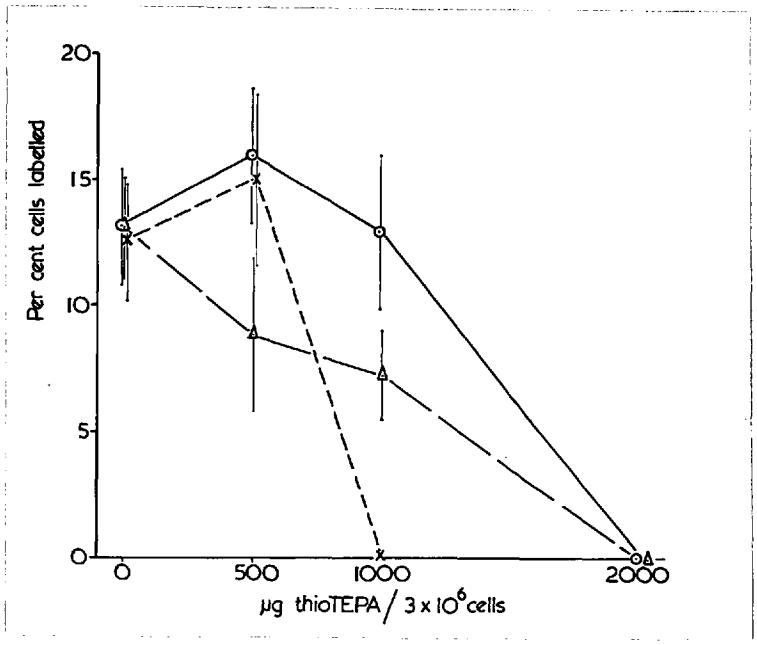
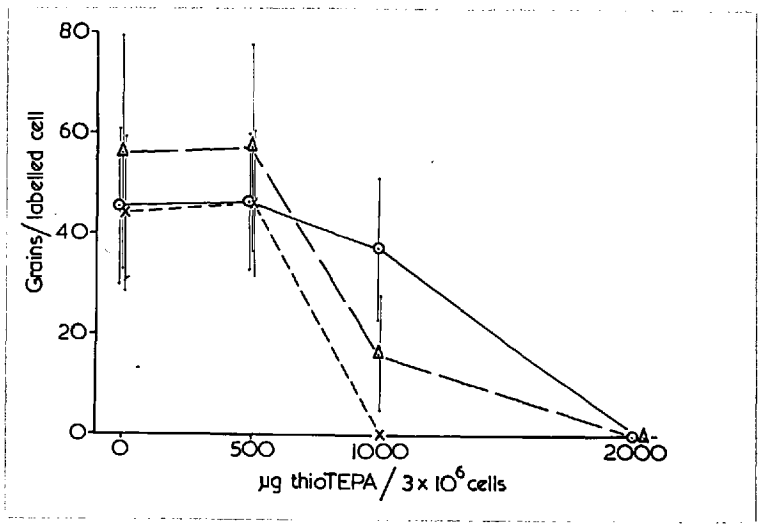


Fig. 17. The effect of nitrogen mustard (diluted in water) on thymidine-H^3 uptake by mouse thymocytes <u>in vitro</u>.





o——o No pretreatment x- - -x ½ hr. pretreatment

Δ— —Δ ¾ hr. pretreatment ——— Standard deviation

Fig. 18. The effect of thioTEPA on thymidine-H^3 uptake by mouse thymocytes <u>in vitro</u>.

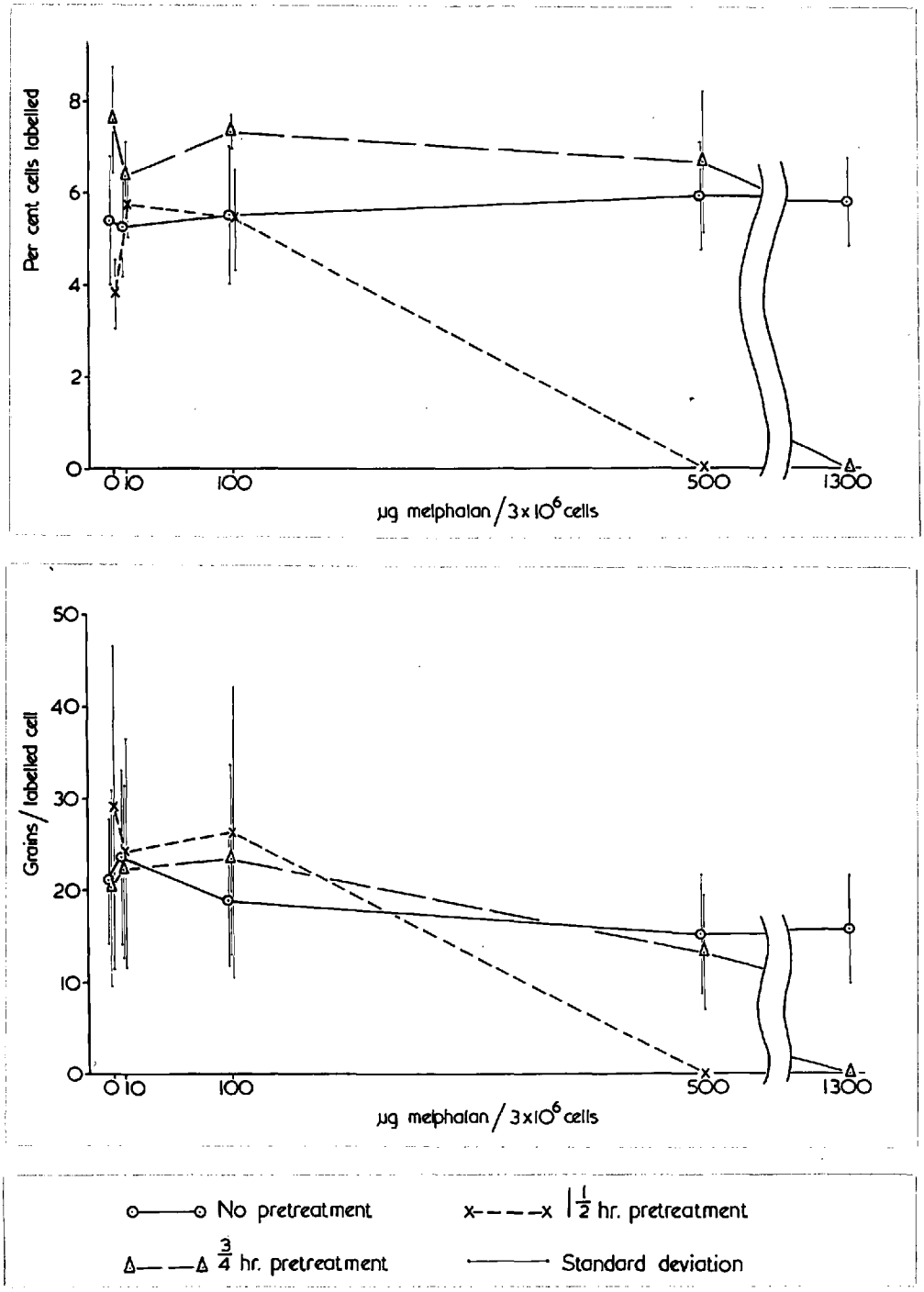


Fig. 19. The effect of melphalan on thymidine-H^3 uptake by mouse thymocytes <u>in vitro</u>.

Cause of the Pretreatment Effect

1. Time lag before the damage becomes detectable. If a certain time has to elapse between the drug effect on the target site(s) and appearance of demonstrable damage, then the longer the drug is allowed to act on the cell before the damage is assessed, the greater the amount of damage likely to be recorded. If the drug action occurs relatively quickly but damage does not become detectable until some time later, then the same damage should result whether the cells are in contact with the drug throughout the pretreatment period or whether the cells are exposed to drug for the initial part of the pretreatment period followed by incubation in drug-free medium for the rest of the period. An experiment along these lines was performed on thymocytes (3×10^6 cells/ml.) from a 24 g. female A2G mouse in medium containing pooled A2G mouse serum. Nitrogen mustard (10 μg./3×10^6 cells) was allowed to act on 1 ml. aliquots of the cell suspension for 30 min. The drug was removed from the cells by centrifuging the tubes at 650 g. and 4°C for 5 min., after which the supernate was discarded and replaced by 1 ml. enriched medium. Incubation was then continued at 37°C. Thymidine-H^3 (0.1 μc in 0.1 ml.) was added to one tube immediately after the fresh medium, to a second ½ hr. after the fresh medium and to a third one hour after the fresh medium. The uptake of thymidine-H^3 by cells in these tubes was compared with that by cells treated continuously with nitrogen mustard for 30, 60 or 90 min. before

TABLE VII. THE EFFECT ON LABELLING OF A TIME LAG AT 37° C BETWEEN THE INCUBATION OF MOUSE THYMOCYTES WITH NITROGEN MUSTARD AND THE ADDITION OF THYMIDINE-H^3

	Grains per labelled cell	Percentage of cells labelled
HN2 ↓ 30 min. ═══ ┘ Th-H^3 ↓ 30 min. └	22.97	4.0
HN2 ↓ 30 min. ═══ ┘ 30 min. Th-H^3 ↓ 30 min. └	29.37	7.6
HN2 ↓ 30 min. ═══ ┘ 60 min. Th-H^3 ↓ 30 min. └	31.33	7.0
HN2 ↓ 60 min. ═══ ┘ Th-H^3 ↓ 30 min. └	9.0	2.4
HN2 ↓ 90 min ═══ ┘ Th-H^3 ↓ 30 min. └	Unlabelled	Unlabelled
Water ↓ 30 min. +++ ┘ Th-H^3 ↓ 30 min. └	34.5	6.6
Water ↓ 30 min. +++ ┘ 30 min. Th-H^3 ↓ 30 min. └	35.87	6.8
Water ↓ 30 min. +++ ┘ 60 min. Th-H^3 ↓ 30 min. └	49.2	7.6

═══ Indicates nitrogen mustard in the medium

┘ └ Indicates replacement of supernatent fluid, after centrifugation, by fresh nutrient medium (1 ml.)

+++ Indicates water in the medium

Th-H^3 = Addition of thymidine-H^3

HN2 = Addition of nitrogen mustard

removal of the drug and incubation in fresh enriched medium and thymidine-H^3. Thirty min. incorporation time was allowed for the thymidine-H^3.

From the results given in Table VII, drug damage appears to be related to the time of contact with the drug and not to the time between the addition of drug and the addition of thymidine-H^3. A pretreatment period of 90 min. prevented labelling with thymidine-H^3 if the cells were in contact with nitrogen mustard throughout the pretreatment period, while a pretreatment period of the same time but with the cells in contact with drug for only 30 min. had no inhibitory effect on labelling. Increasing the lag period between drug treatment with nitrogen mustard and incubation with thymidine-H^3 resulted in a slight increase in grain count. This could have been due to some recovery from drug damage taking place but is more likely to be due to recovery from cooling after centrifugation. The cells with a lag after drug treatment would have had time to return to 37°C before the thymidine-H^3 was added. Without a lag after centrifugation, the cells would have still been cold when the thymidine-H^3 was added and decreased incorporation is, therefore, not surprising. A similar effect occurred in the water controls (in which 0.1 ml. water was added instead of 0.1 ml. nitrogen mustard).

2. Continuous and cumulative damage. If nitrogen mustard needs to be in contact with the cells throughout the pretreatment period

TABLE VIII. THE ALKYLATING ABILITY OF NITROGEN MUSTARD AND CHLORAMBUCIL AFTER 1½ HR. INCUBATION WITH CELLS IN NUTRIENT MEDIUM

Initial drug concentration (µg./ml. cell suspension)	Absorbance at 565 µ	
	Incubation time at 37° C	
	0 hr.	1½ hr.
Chlorambucil		
100	1.35	0.69
Nitrogen mustard		
10	0.45	0.33) duplicate 0.37) readings
2	0.22	Not determined

to produce the full pretreatment effect, then, unless recovery is postulated, drug damage to the cells must be continuous and cumulative. According to this hypothesis, the drug would tend to be relatively ineffective after a short pretreatment period because the drug would not have had time to damage the cell sufficiently to interfere with its ability to incorporate thymidine-H^3, and the differences between the extent of the pretreatment effect produced by the various alkylating agents would depend on the rate of attack by the drug.

If continuous and cumulative damage to the cell is the cause of the pretreatment effect shown by nitrogen mustard, one would expect active drug to remain in the medium in the presence of cells and serum for at least 1½ hr. Goldenberg and Alexander (1965) state that half the alkylating potential of nitrogen mustard is lost after 75 min. at 37°C in tissue culture medium containing horse serum. If the drug reacts in the present experimental conditions in the same manner, then much active drug would remain after 1½ hr. incubation. To check this, the alkylating potential of nitrogen mustard and chlorambucil was estimated after 0 and 1½ hr. incubation at 37°C in nutrient medium containing mouse thymocytes (3×10^6 cells/ml.). The method used was that described by Boyland, Staunton and Williams (1961).

As seen from the figures (Table VIII), a definite alkylating reaction can be detected for both nitrogen mustard (initially 10 µg.)

and chlorambucil (initially 100 μg.) after 1½ hr. incubation at 37°C with cells in nutrient medium. No attempt was made to determine the amount of drug remaining after this time though it can be seen from the figures that the amount of nitrogen mustard left was more than 2 μg./ml. nutrient medium. The drawback to this method of estimating alkylating ability is that it does not distinguish between the unreacted difunctional agent and the monofunctional compound formed by partial reaction.

In spite of the caution needed in the interpretation of the estimations of the alkylating ability of these drugs after 1½ hr. in solution since some of the alkylating ability detected would have been due to partially reacted drug, it seems likely that active nitrogen mustard and chlorambucil would remain after this time. It is therefore feasible to suggest that nitrogen mustard might have damaged the cells continuously throughout 1½ hr. and that the pretreatment effect was due to the cumulative effect of this damage. This hypothesis would explain the need for the drug to be in contact with the cells throughout the pretreatment period to produce the full pretreatment effect. The longer the cells were in contact with the drug, the greater the damage and the lower the thymidine-H^3 uptake.

Although decreased thymidine-H^3 uptake in the presence of these drugs has been demonstrated, this does not necessarily imply that DNA synthesis was specifically affected. Other types of injury to

the cell might produce the same result. The possible specificity of the action of nitrogen mustard against DNA synthesis has been discussed above (page 20-1). Some authors state that RNA synthesis is affected to the same extent as DNA synthesis while others state that some drug concentrations will inhibit DNA synthesis but allow RNA synthesis to continue at the normal rate. Uridine-H^3 uptake in the presence of various alkylating agents was followed in this cell system to determine whether the damage being measured by following the inhibition of thymidine-H^3 uptake was specific to DNA. It is possible that the cells failed to incorporate thymidine-H^3 because they were dead before they came into contact with the thymidine-H^3 and that non-specific damage unrelated to inhibition of nucleic acid synthesis was being followed. The uptake of tritiated amino acids was therefore examined in the presence of these drugs in an attempt to determine what type of damage to the cells was being measured.

Effect of Selected Agents on Uridine-H^3 Uptake by Mouse Thymocytes in vitro

Nitrogen mustard. To determine whether nitrogen mustard was acting selectively against DNA synthesis, the effect of this drug on uptake of uridine-H^3 into mouse thymocytes was determined with the same experimental procedure as that described above to show the effects of alkylating agents on thymidine-H^3 uptake. Thymocytes from a 32.6 g. female A2G mouse were used at a concentration of

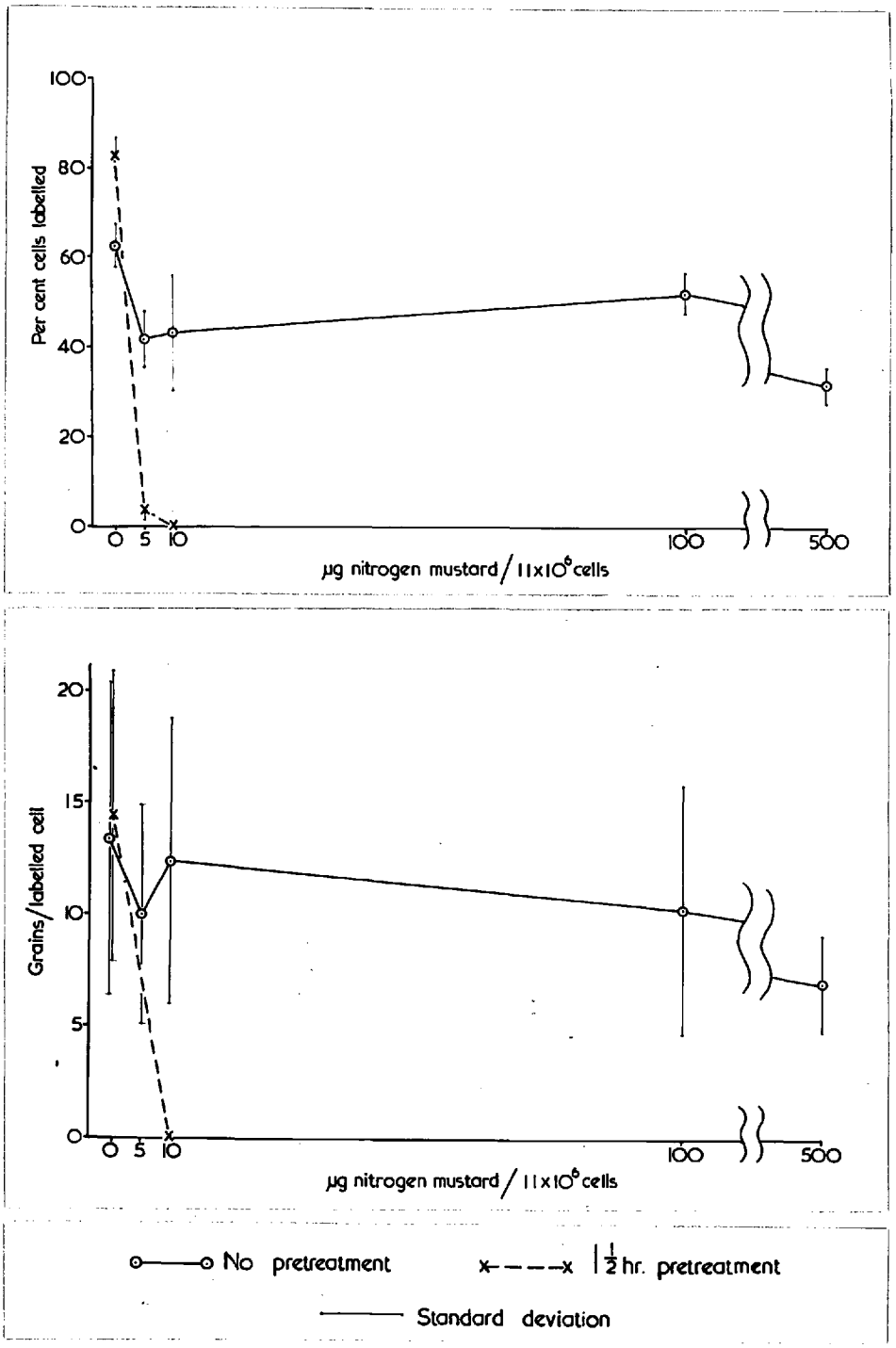


Fig. 20. The effect of nitrogen mustard on uridine-H^3 uptake by mouse thymocytes <u>in vitro</u>.

10.6×10^6 cells/ml. nutrient medium. Pyrogen-free distilled water was used as the drug diluent and added to the control tubes. Uridine-H^3 was added to the control or drug-treated cells in a volume of 0.1 ml. (containing 1 μc.) after 0, ¾ or 1½ hr. pretreatment of the cells with the drug or water, and 35 min. incorporation time was allowed for the uridine-H^3 before the labelling was stopped.

With no pretreatment, 500 μg. nitrogen mustard/10.6×10^6 cells decreased the proportion of cells labelled with uridine-H^3 to about 50 per cent of the control value (Fig. 20, Appendix Table 17). After 1½ hr. pretreatment, however, cells treated with 10 μg. nitrogen mustard showed no detectable uptake of uridine-H^3. While some of the cells treated with 5 μg. nitrogen mustard for 1½ hr. were definitely labelled (3.5%), the number of grains over the labelled cells was very close to the arbitrary 4 grains background level and the mean value was therefore not determined. No attempt was made to distinguish between uridine-H^3 incorporated into RNA and any which may have been incorporated into DNA. The effect of nitrogen mustard on uptake of uridine-H^3 (whether into RNA alone or into DNA as well as RNA) is very similar to the drug effect on thymidine-H^3 incorporation, with 5 μg. reducing uptake of these two nucleosides to negligible proportions after 1½ hr. pretreatment.

Melphalan. A cell suspension was prepared from the thymus of a 19.9 g. female A2G mouse and was adjusted to a concentration of 3×10^6 cells/ml. nutrient medium. The cells were incubated in the

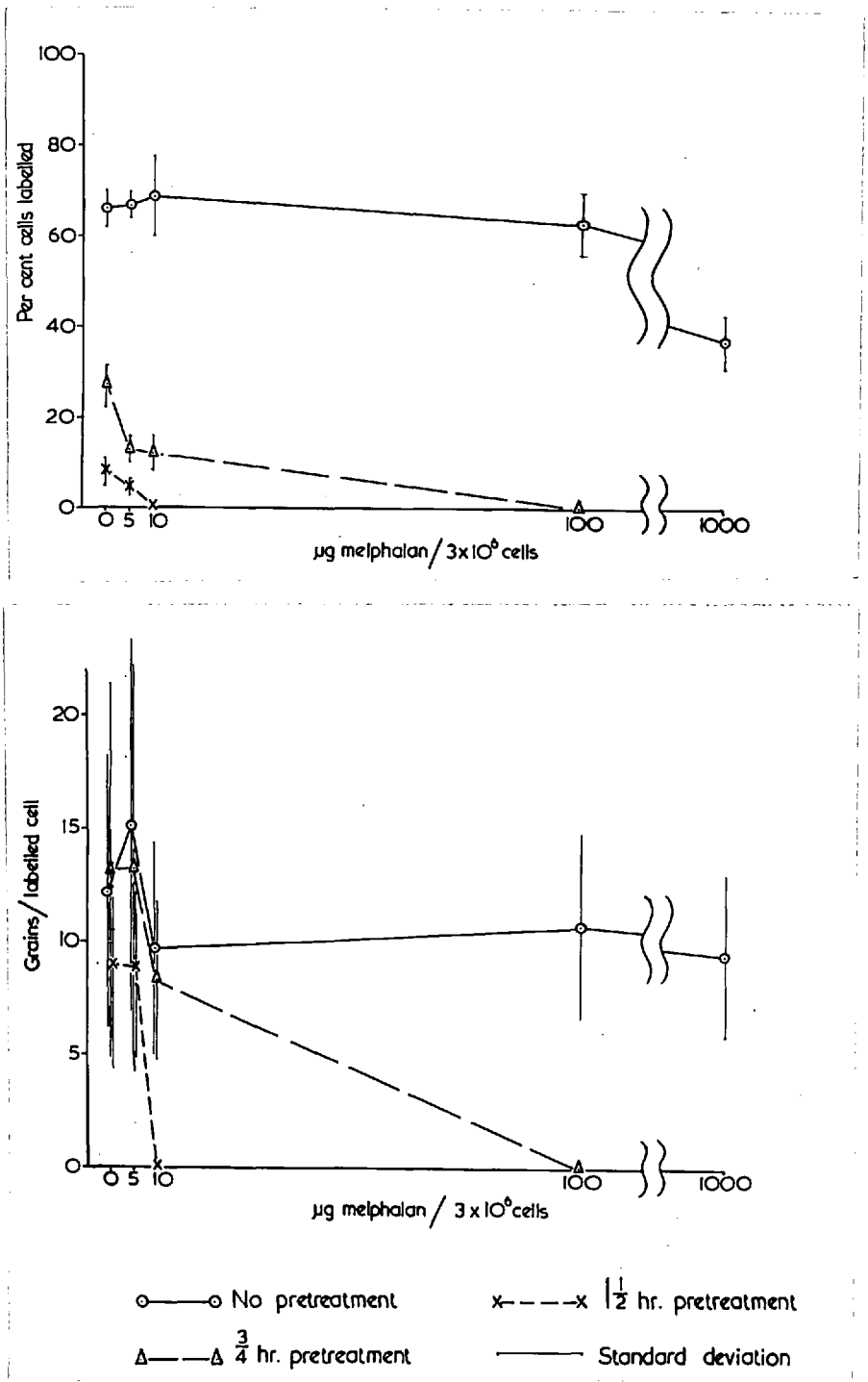


Fig. 21. The effect of melphalan on uridine-H^3 uptake by mouse thymocytes <u>in vitro</u>.

presence of melphalan or melphalan diluent for 0, ¾ or 1½ hr. before their ability to take up uridine-H^3 in a 35 min. incubation period was assessed. Without pretreatment, 1000 µg. melphalan had little effect on the number of grains over each labelled cell but reduced the proportion of labelled cells to about 50 per cent of the control value (Fig. 21, Appendix Table 18). Uridine-H^3 uptake was, however, undetectable after ¾ hr. pretreatment with 100 µg. melphalan and after 1½ hr. pretreatment with 10 µg. melphalan. Superficially, this is similar to the pronounced pretreatment effect noticed when the effect of melphalan on thymidine-H^3 uptake was examined. But, since a pretreatment effect was found in the diluent controls (66% cells labelled without pretreatment, 27% labelled after ¾ hr. pretreatment and only 8% after 1½ hr. pretreatment), the enhanced drug effect after pretreatment is probably as much due to the presence of the drug diluent as to the presence of melphalan itself.

Chlorambucil. The effect of chlorambucil on uridine-H^3 uptake was examined in a cell suspension containing 9.4 x 10^6 cells/ml. nutrient medium and made from the thymus of a 21.7 g. female A2G mouse. As with melphalan, uridine-H^3 uptake, after pretreatment of the cells with chlorambucil, was affected more by the presence of the diluent than by the drug itself. In the diluent controls, 69.2 per cent of the cells were labelled without pretreatment, whereas after 1½ hr. pretreatment only 16.7 per cent were labelled (Fig. 22, Appendix Table 19). Without pretreatment, 500 µg. caused

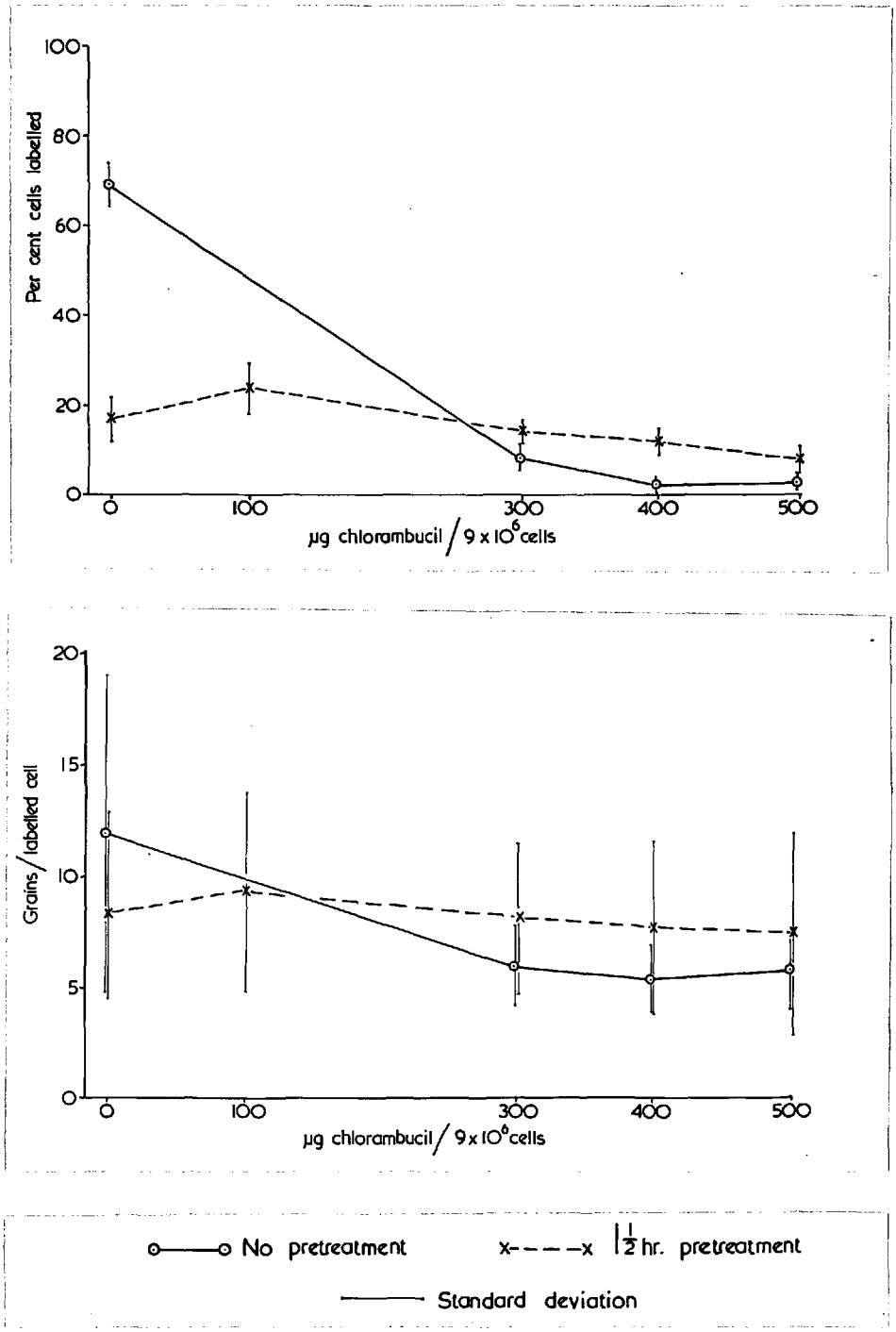


Fig. 22. The effect of chlorambucil on uridine-H^3 uptake by mouse thymocytes <u>in vitro</u>.

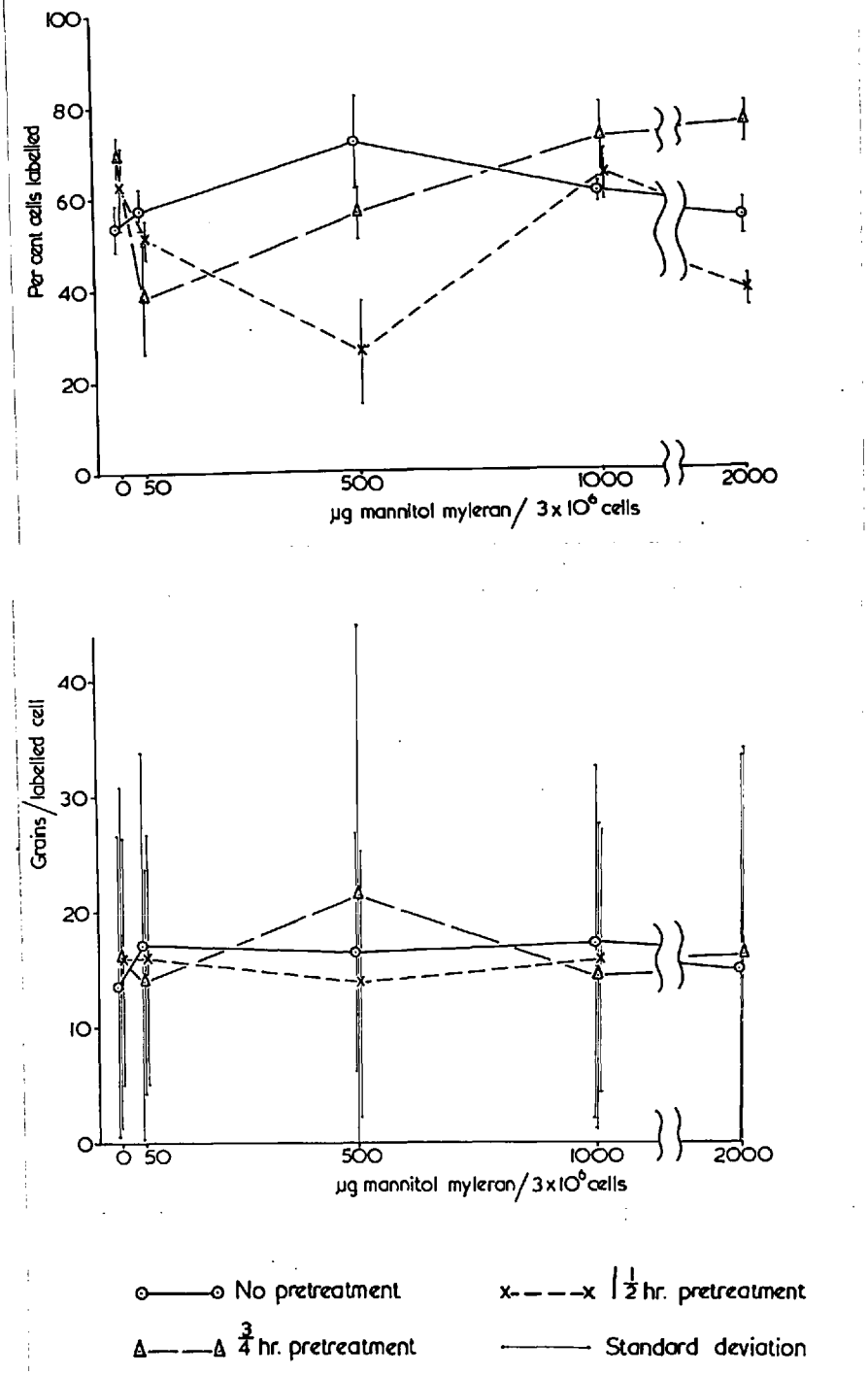


Fig. 23. The effect of mannitol myleran on uridine-H^3 uptake by mouse thymocytes <u>in vitro</u>.

a considerable reduction in uptake of uridine-H^3 but failed to prevent labelling.

Mannitol myleran. The thymus from a 15.8 g. female A2G mouse was made into a cell suspension containing 3 x 10^6 cells/ml. nutrient medium. No inhibition of uridine-H^3 uptake was detected even after the cells had been pretreated with 2 mg. mannitol myleran (Fig. 23, Appendix Table 20).

Summary of Drug Effects on Uridine-H^3 Uptake

Estimating damage caused by cytotoxic agents by following the uptake of thymidine-H^3 by drug-treated cells has the disadvantage of providing no information about any possible drug effects on cells not synthesising DNA during the time of contact with the thymidine-H^3. Drug effects on uptake of uridine-H^3 were followed in the hope of obtaining some information about RNA synthesis in drug-treated cells. The results were disappointing in that, although most nucleated cells are capable of RNA synthesis, the extent of incorporation of uridine-H^3 into mouse thymocytes was variable, with 54 to 85 per cent of the cells being labelled after 35 min. incubation with this metabolite. Further, this metabolite can be incorporated into either RNA or, after methylation to thymidine-H^3, into DNA. In the absence of cytotoxic agents, uridine-H^3 is likely to be incorporated mainly into RNA during 35 min. incubation time. After drug treatment, however, blockage of one synthetic pathway might have influenced the rate of uridine-H^3 uptake via an alternative pathway. For this reason,

labelling with uridine-H^3 is not referred to as RNA synthesis but as uridine-H^3 uptake.

It was found that nitrogen mustard had a similar effect on uridine-H^3 uptake as on thymidine-H^3 uptake, with 5 μg. and 1½ hr. pretreatment reducing labelling to negligible proportions. Mannitol myleran (2 mg.) had no effect on uridine-H^3 uptake even after 1½ hr. pretreatment. Although the effects of chlorambucil and melphalan on uridine-H^3 uptake were obscured by the toxicity of the diluents, uridine-H^3 uptake was reduced in the presence of these two drugs without pretreatment. It can be concluded that nitrogen mustard, melphalan and chlorambucil do not produce a selective effect on DNA synthesis.

Effect of Methotrexate on Uptake of Thymidine-H^3 and Uridine-H^3 by Mouse Thymocytes in vitro

Antifolates are known to have a different mode of action from the alkylating agents. Although a detailed study of the antifolates is outside the scope of this thesis, it was thought that a brief comparison of the effects of one antifolate, methotrexate, with the effects of the alkylating agents might be of interest.

The thymus from a 28.5 g. male (A2G x C57Bl)F_1 hybrid mouse was made into a cell suspension containing 2.7×10^6 cells/ml. nutrient medium. Thymidine-H^3 uptake by these cells in a 35 min. incorporation period was determined after 0 and 1½ hr. pretreatment with methotrexate or methotrexate diluent. Up to 100 μg. methotrexate had no effect on

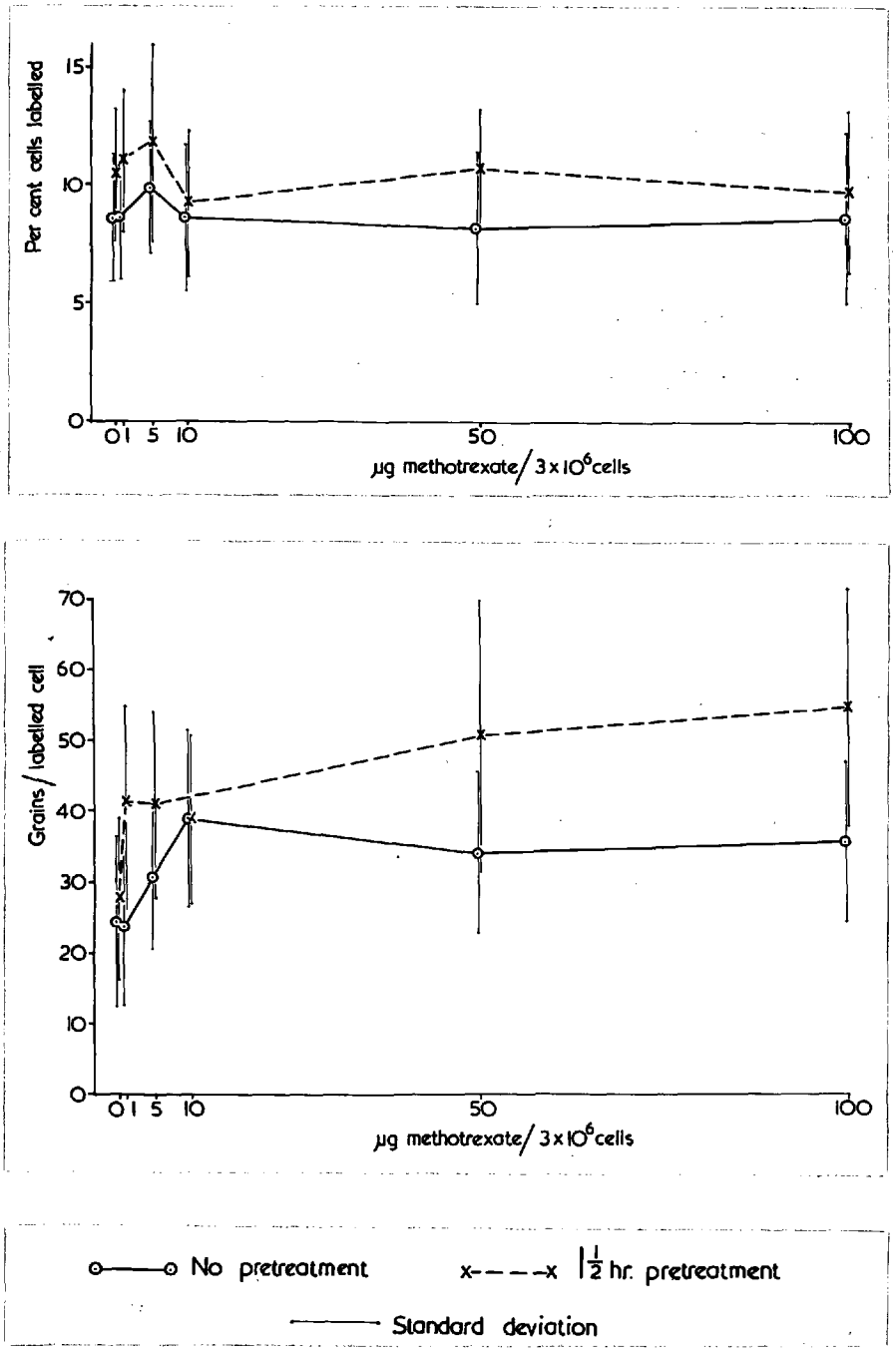
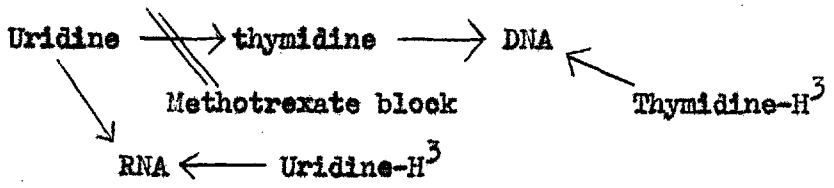


Fig. 24. The effect of methotrexate on thymidine-H^3 uptake by mouse thymocytes <u>in vitro</u>.

the percentage of cells labelled with thymidine-H^3 though there was a marked effect on the uptake of thymidine-H^3 by individual cells (Fig. 24, Appendix Table 21). After $1\frac{1}{2}$ hr. pretreatment with 100 µg. methotrexate, the mean grain count per labelled cell was double the value for the diluent control. In an untreated cell, uridine is methylated to thymidine and this thymidine is utilised for DNA synthesis. Methotrexate is known to cause a block in the uridine to thymidine pathway by interfering with the action of dihydrofolic acid reductase (O'Brien, 1962). Since fresh thymidine cannot be synthesised within the cell in the presence of this drug, more thymidine-H^3 than the control quantity would be taken up from this medium to compensate for this block. The increased uptake of thymidine-H^3 in the presence of methotrexate is less pronounced without pretreatment than after $1\frac{1}{2}$ hr. pretreatment. This is presumably because some reserve of thymidine is initially available within the cell for DNA synthesis and this reserve becomes depleted during the pretreatment period.



The effect of methotrexate on uridine-H^3 uptake by the same concentration of cells (2.7×10^6 cells/ml. nutrient medium) was determined with thymocytes taken from a 20.0 g. male (A2G x C57Bl)F_1

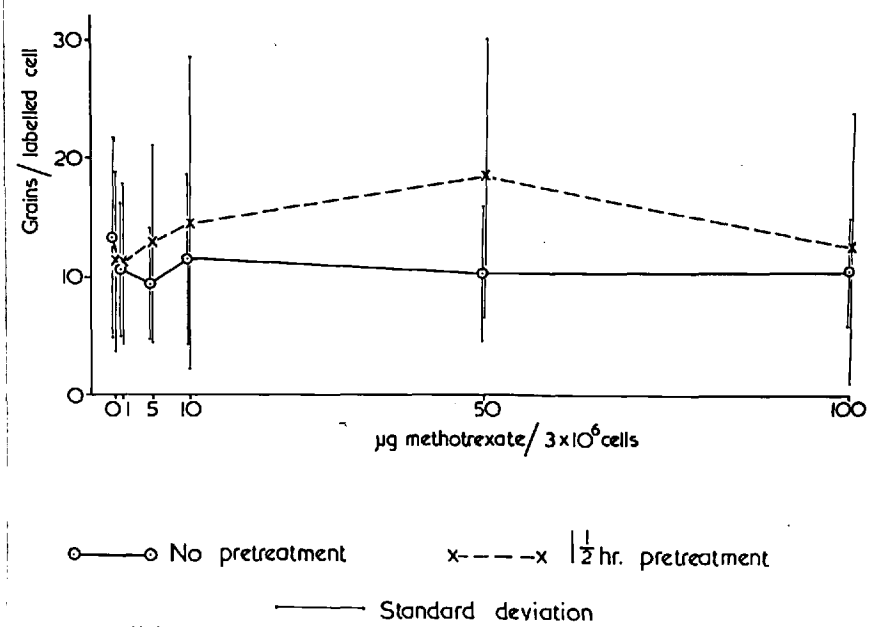


Fig. 25. The effect of methotrexate on uridine-H^3 uptake by mouse thymocytes <u>in vitro.</u>

hybrid mouse. Methotrexate (100 μg.) had no effect on the mean number of grains over labelled cells, indicating that methotrexate has no effect on uridine-H^3 incorporation by individual cells (Fig. 25, Appendix Table 22). These results support the view that this drug causes a block in the uridine to thymidine pathway. The significance of these results is discussed on page 146.

Comparative Effects of Alkylating Agents on Tyrosine-H^3 Uptake by Mouse Thymocytes in vitro

Since tyrosine-H^3 can be taken up by 99 per cent of the cells from the mouse thymus (slightly less after ½ hr. incubation), it was hoped that following the uptake of this metabolite by drug-treated cells might indicate the mode of action of the cytotoxic agents on non-dividing cells.

Female A2G mice, weighing 14.8 to 17.5 g., were used and the thymuses were teased in nutrient medium containing mouse serum to provide cell suspensions containing 3×10^6 cells/ml. The experimental plan was the same as that used for the thymidine-H^3 studies. The ability of the cells to incorporate tyrosine-H^3 (1 μc final concentration) was determined after 0, ¾ and 1½ hr. pretreatment with freshly-dissolved drug or diluent. An incorporation time of 35 min. was allowed for the tyrosine-H^3 in each case.

Nitrogen mustard. As with thymidine-H^3, tyrosine-H^3 uptake in the presence of nitrogen mustard depended on the drug concentration and on the pretreatment time. Thus, treating the cells with 10 μg.

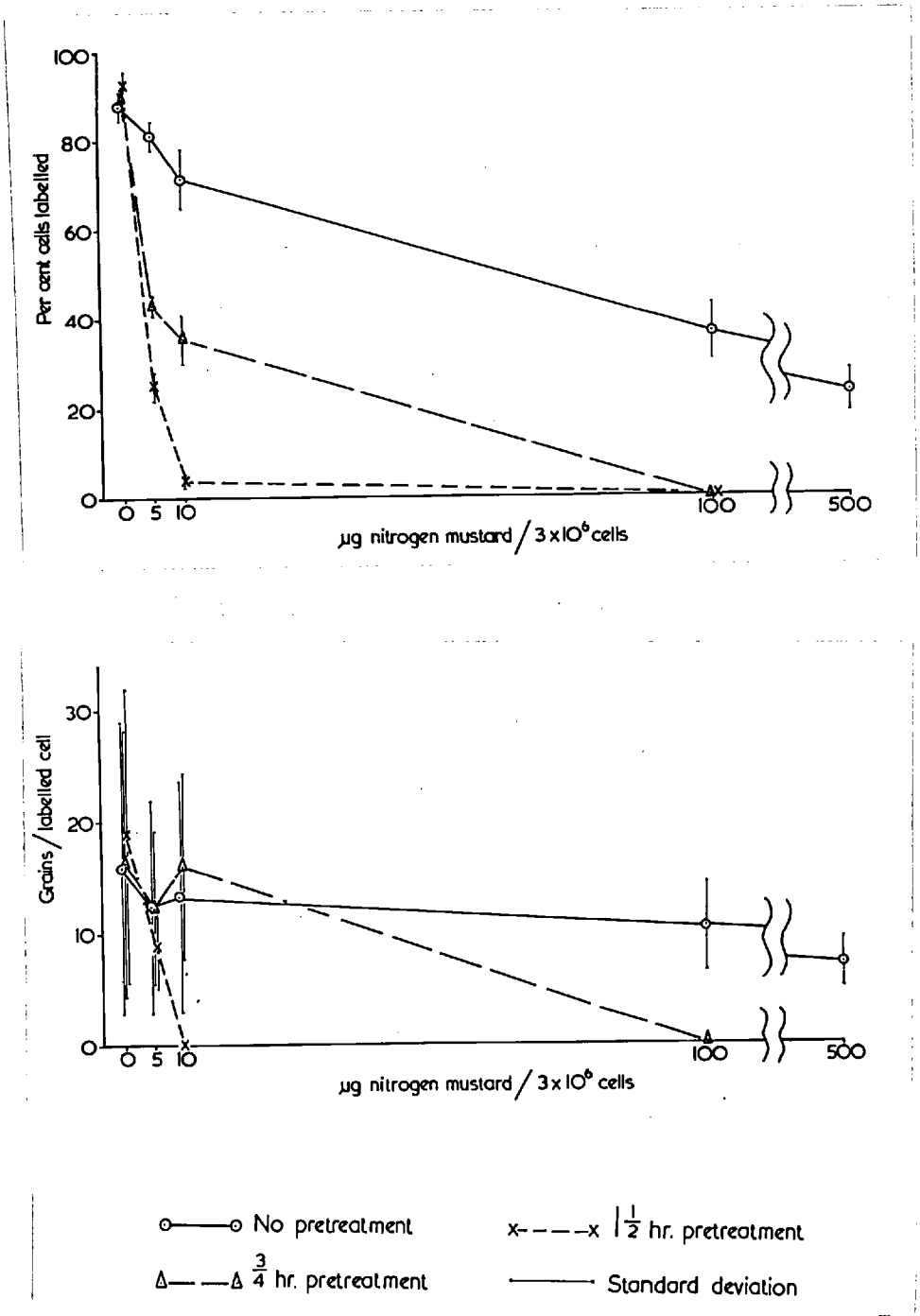


Fig. 26. The effect of nitrogen mustard on tyrosine-H^3 uptake by mouse thymocytes <u>in vitro</u>.

nitrogen mustard immediately before the addition of tyrosine-H^3 resulted in little change from the control value in the percentage of cells labelled (71% labelled instead of 88%), whereas there was negligible uptake of label after 1½ hr. pretreatment with this drug concentration (Fig. 26, Appendix Table 23). There was little difference, after 1½ hr. pretreatment, between the concentration of nitrogen mustard needed to inhibit uptake of tyrosine-H^3 (10 μg.) and that needed to inhibit uptake of thymidine-H^3 and uridine-H^3 (5 μg.) after the same pretreatment time.

ThioTEPA. Without pretreatment, 2 mg. thioTEPA reduced the uptake of tyrosine-H^3 to negligible proportions, and after 1½ hr. pretreatment, 1 mg. prevented uptake (Fig. 27, Appendix Table 24). Thus, thioTEPA produced a moderate pretreatment effect on tyrosine-H^3 uptake similar to its effect on thymidine-H^3 uptake.

Tretamine. The lowest concentration of tretamine which inhibited thymidine-H^3 uptake after 1½ hr. pretreatment (1 mg.) had a slight effect on the percentage of cells labelled with tyrosine-H^3 but had no effect on the mean number of grains over the labelled cells after this pretreatment time (Fig. 28, Appendix Table 25).

Mannitol myleran had no effect on uptake of tyrosine-H^3 by mouse thymocytes even when the cells were pretreated with 2 mg./ 3×10^6 cells for 1½ hr(Fig. 29, Appendix Table 26).

Melphalan. In the presence of the melphalan diluent (0.1 ml. added to 1 ml. of cell suspension), very few cells became labelled

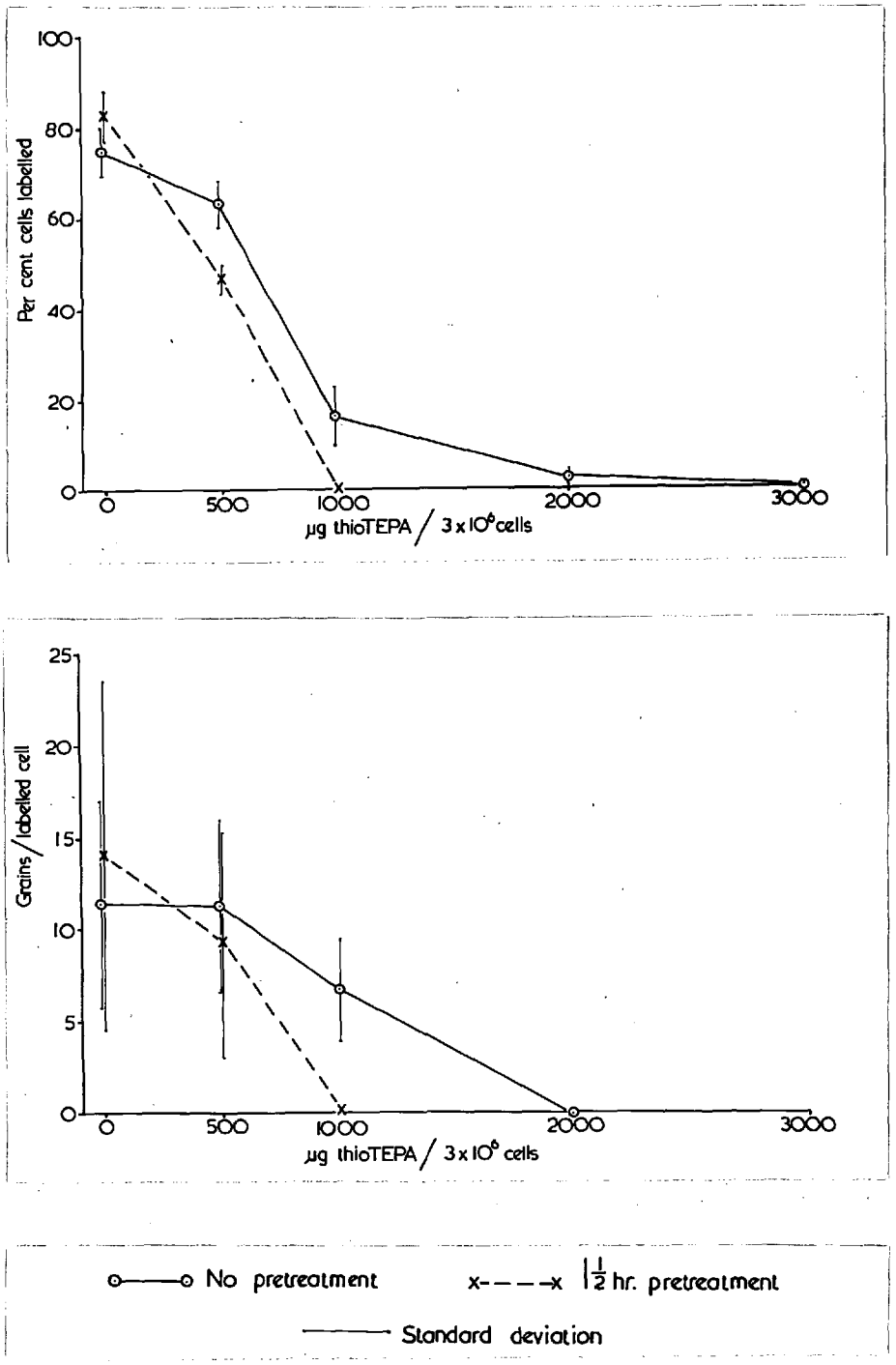


Fig. 27. The effect of thioTEPA on tyrosine-H^3 uptake by mouse thymocytes <u>in vitro</u>.

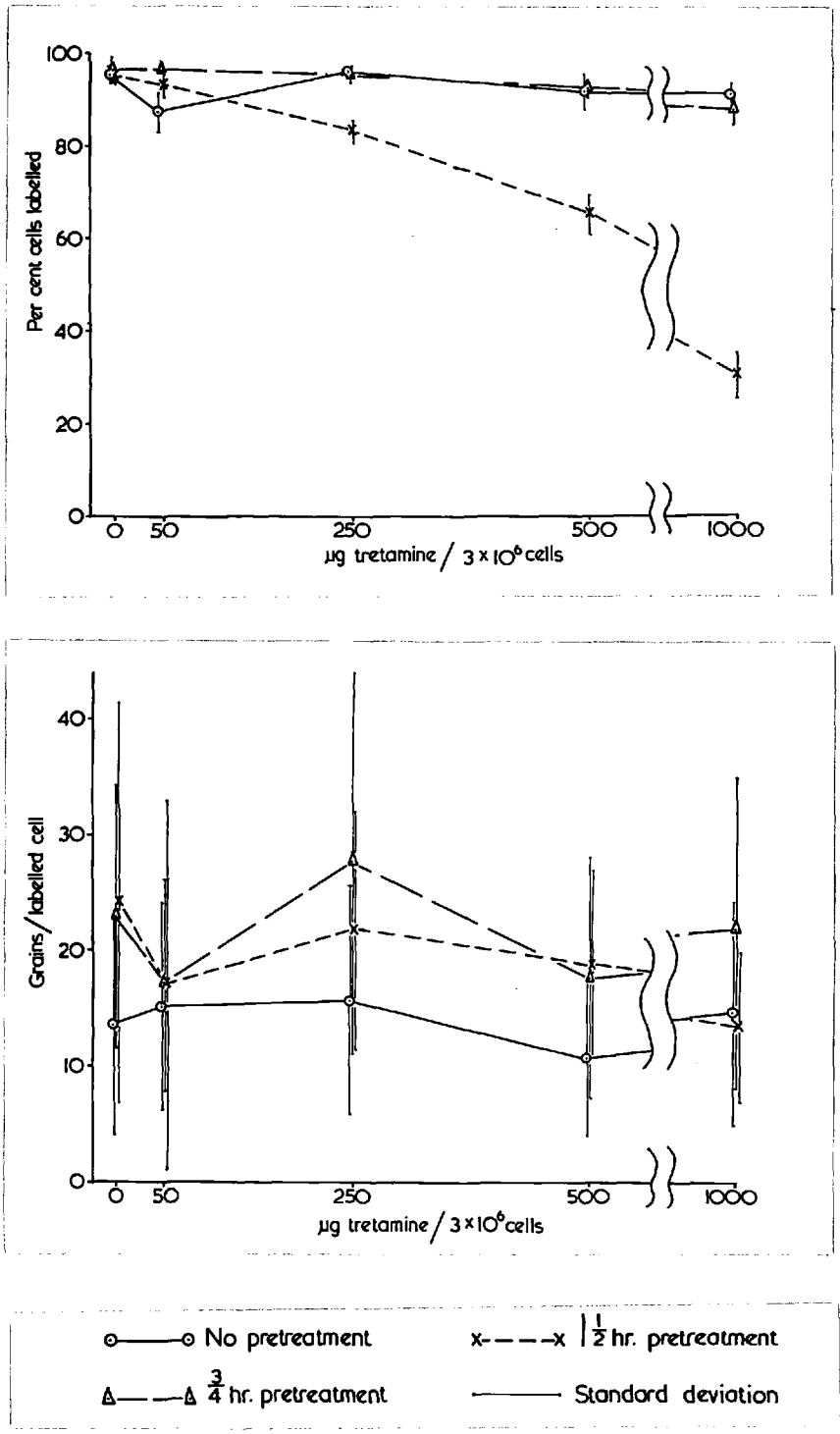


Fig. 28. The effect of tretamine on tyrosine-H^3 uptake by mouse thymocytes <u>in vitro</u>.

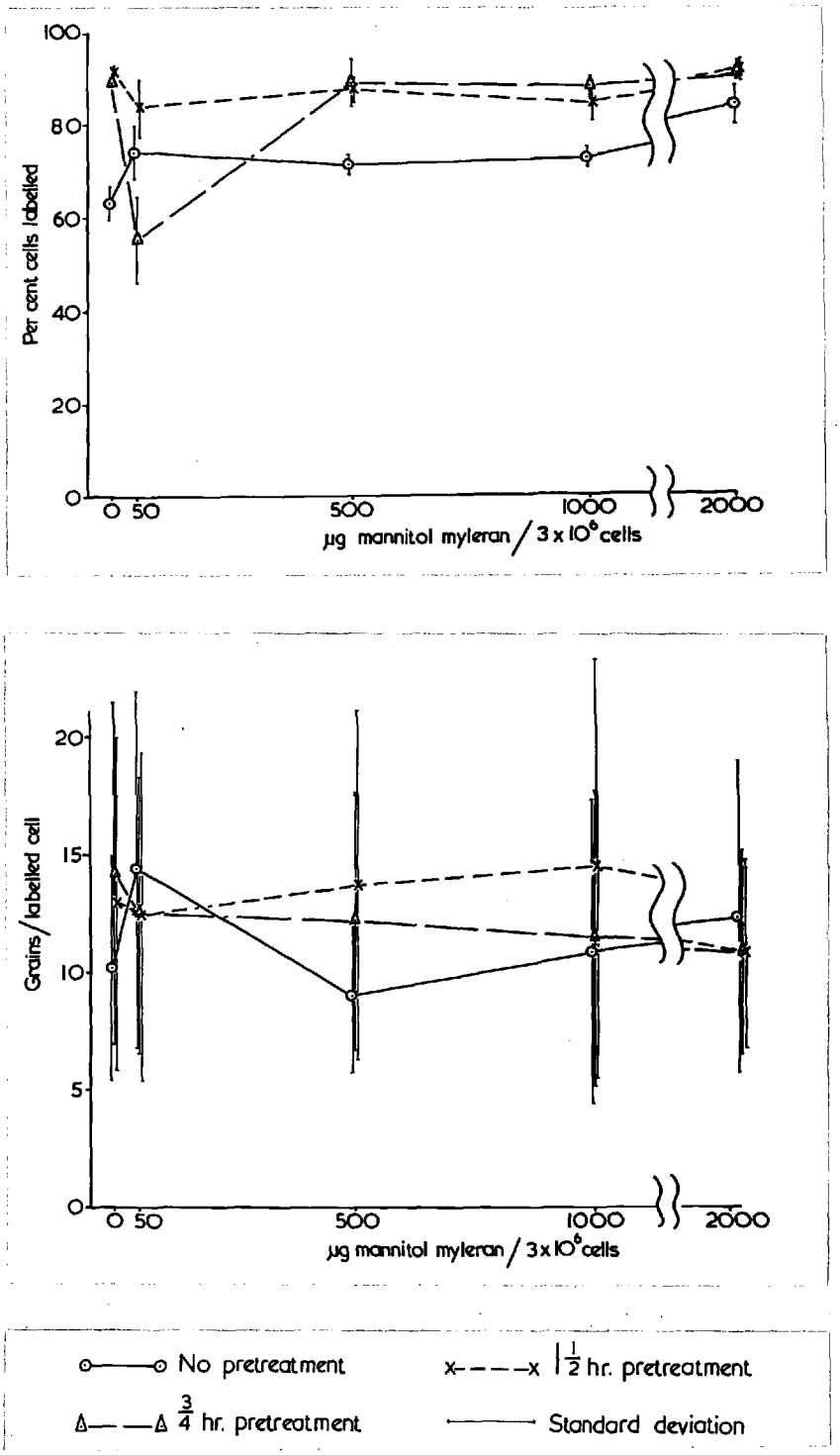
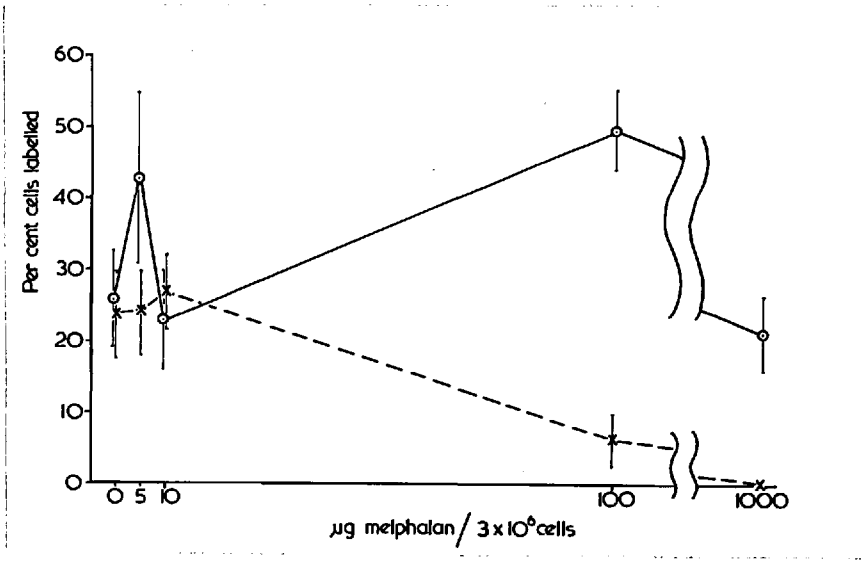
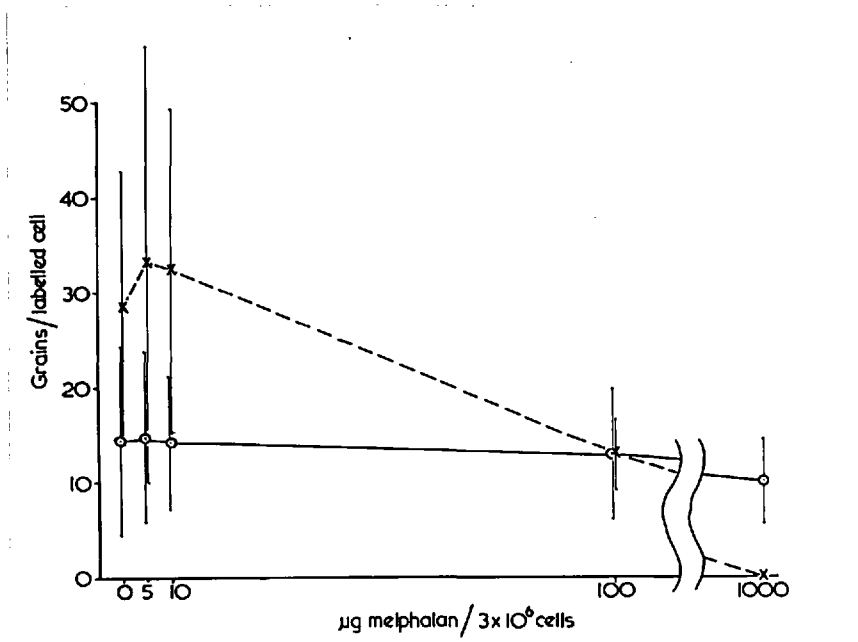


Fig. 29. The effect of mannitol myleran on tyrosine-H^3 uptake by mouse thymocytes <u>in vitro</u>.





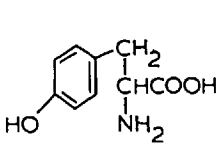
⊙——⊙ No pretreatment x- - - -x ½ hr. pretreatment

——— Standard deviation

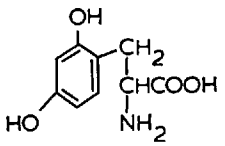
Fig. 30. The effect of melphalan on the uptake of tyrosine-H^3 (5 µc/ml.) by mouse thymocytes <u>in vitro</u>.

in a 35 min. incorporation period with tyrosine-H^3. Without pretreatment, only 8.4 per cent of the cells were labelled and with ¾ and 1½ hr. pretreatment this value was as low as 1.8 and 2.8 per cent cells labelled respectively. Labelling was so low on smears of drug-treated cells that the reduction in uptake of the label was probably as much due to the presence of the diluent as to the melphalan itself. In the hope of obtaining higher grain counts, the experiment was repeated with a higher concentration of tyrosine-H^3 (5 μc final concentration instead of 1 μc).

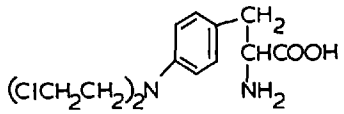
The thymus was taken from a 20.3 g. female A2G mouse and the thymocyte suspension was used at a concentration of 3×10^6 cells/ml. nutrient medium. Even with the higher concentration of tyrosine-H^3, labelling in the diluent controls was far below the level usually attained in the water controls, with only 25.9 per cent cells being labelled without pretreatment and 23.6 per cent after 1½ hr. pretreatment (Fig. 30, Appendix Table 27). An unusual finding was that in the diluent controls and with 5 μg. and 10 μg. melphalan, the mean number of grains/labelled cell after 1½ hr. pretreatment was about twice the corresponding value without pretreatment. Uptake of tyrosine-H^3 by cells in the presence of 100 μg. melphalan was the same without pretreatment as after 1½ hr. pretreatment, and although 1000 μg. melphalan had no inhibitory effect without pretreatment, this concentration (1000 μg.) prevented labelling after 1½ hr. pretreatment. The effect of melphalan on


CH2
CHCOOH
HO
NH2

IX


OH
CH2
CHCOOH
HO
NH2

X


CH2
CHCOOH
(ClCH2CH2)2N
NH2

XI

uptake of tyrosine-H^3 by thymocytes was slightly less drastic than its effect on uptake of thymidine-H^3.

A possible antimetabolite effect shown by melphalan. A higher tyrosine-H^3 concentration does improve labelling in the presence of the melphalan diluent; but since tyrosine (IX) is structurally very similar to phenylalanine (X), the amount of labelled metabolite might have affected the action of melphalan (XI) (phenylalanine mustard). Tyrosine-H^3 might have been taken up in preference to the cytotoxic agent. This is, however, unlikely since the concentration of melphalan needed to prevent uptake of tyrosine-H^3 after $1\frac{1}{2}$ hr. pretreatment is 1000 µg. (2.62 mM) while the highest concentration of tyrosine-H^3 used was almost a million-fold less (7.46×10^{-6} mM). It is more likely that the converse is true and that the melphalan inhibited tyrosine-H^3 uptake because the phenylalanine part of the melphalan filled the sites normally available for tyrosine-H^3.

To test this possibility, tyrosine-H^3 uptake in the presence of unlabelled phenylalanine was examined, using amounts equivalent to those contained in melphalan. The solid amino acid (54.1 mg.) was dissolved in hydrochloric acid (0.5 ml. 3.6% HCl) and diluted with distilled water to 5.41 mg./ml. Other dilutions were made from this solution, using 0.18% HCl as diluent. The amount of sodium bicarbonate needed to neutralize the acid and restore the pH to 7.4 when 0.1 ml. phenylalanine solution was added to 1 ml. nutrient medium was determined at the beginning of the experiment.

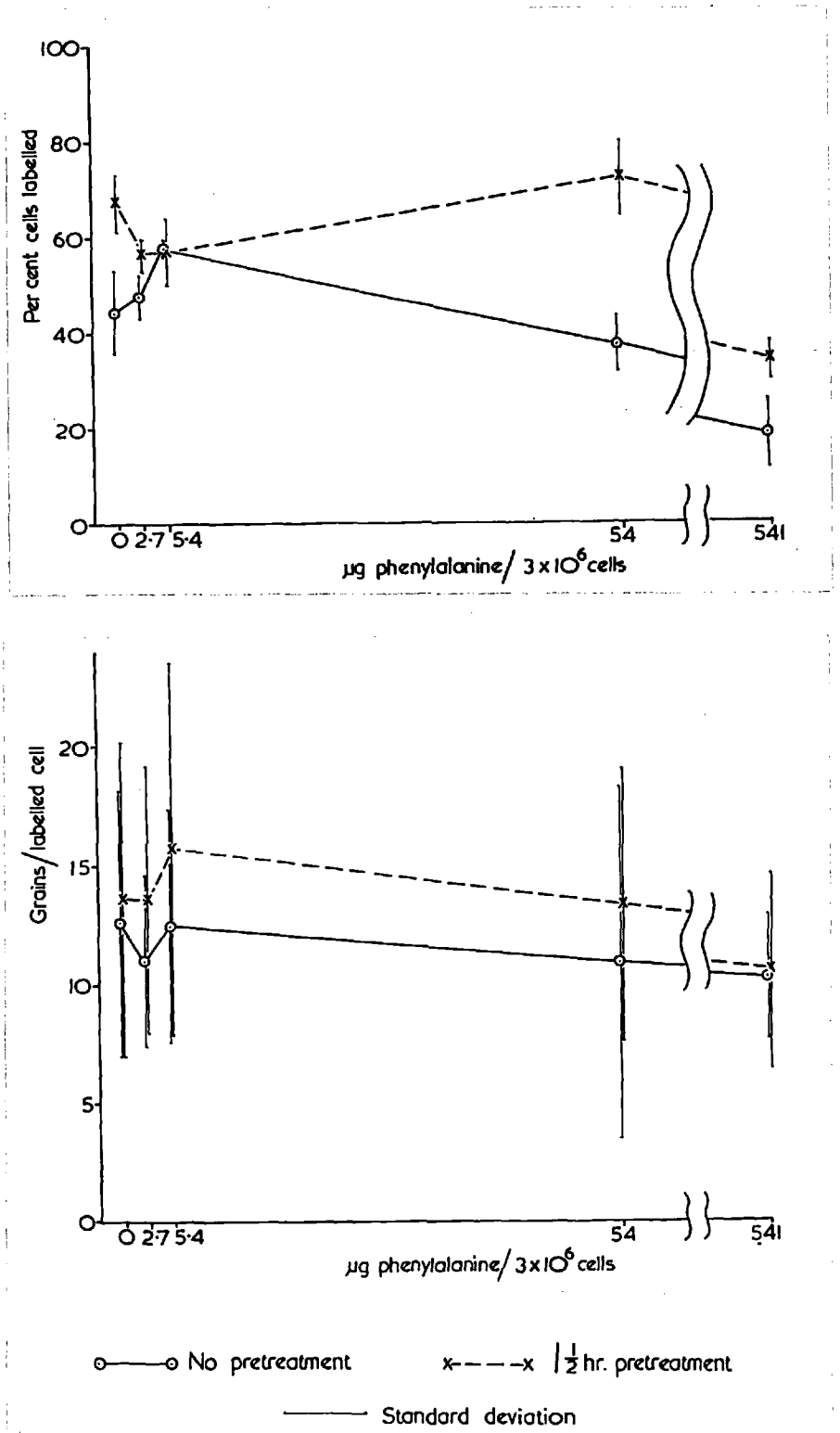


Fig. 31. The effect of phenylalanine on the uptake of tyrosine-H^3 (1 µc/ml.) by mouse thymocytes <u>in vitro</u>.

This quantity (0.05 ml. 5% $NaHCO_3$) was added to each 1 ml. aliquot of cell suspension immediately after the addition of 0.1 ml. phenylalanine solution or diluent. The experimental plan was as usual, the thymus from a 23.5 g. female A2G mouse providing a cell suspension with 3 x 10^6 cells/ml. Uptake of tyrosine-H^3 (1 μc final concentration) by the cells in the presence of phenylalanine or diluent was determined after 0 and 1½ hr. pretreatment. Uptake of leucine-H^3 after 1½ hr. pretreatment with phenylalanine was also followed. From Fig. 31 and Appendix Table 28 it can be seen that the highest concentration of phenylalanine (541 μg. equivalent to 1000 μg. melphalan) reduced the proportion of cells labelled with tyrosine-H^3 to about half the appropriate control value, both without pretreatment and after 1½ hr. pretreatment. There was a negligible effect on the mean grain count/labelled cell.

Leucine-H^3 uptake after 1½ hr. pretreatment was unaffected by 541 μg. phenylalanine (Fig. 32, Appendix Table 29), showing that the reduced tyrosine-H^3 uptake was not due to acidity or general toxicity of the phenylalanine solution.

Since phenylalanine has no effect on leucine-H^3 uptake by mouse thymocytes, even at concentrations which reduce the proportion of cells labelled with tyrosine-H^3 by 50 per cent, the effect of melphalan on leucine-H^3 uptake was determined to find:

(a) whether the phenylalanine mustard would produce an effect;

(b) whether the results would be similar to those found for tyrosine-H^3 uptake.

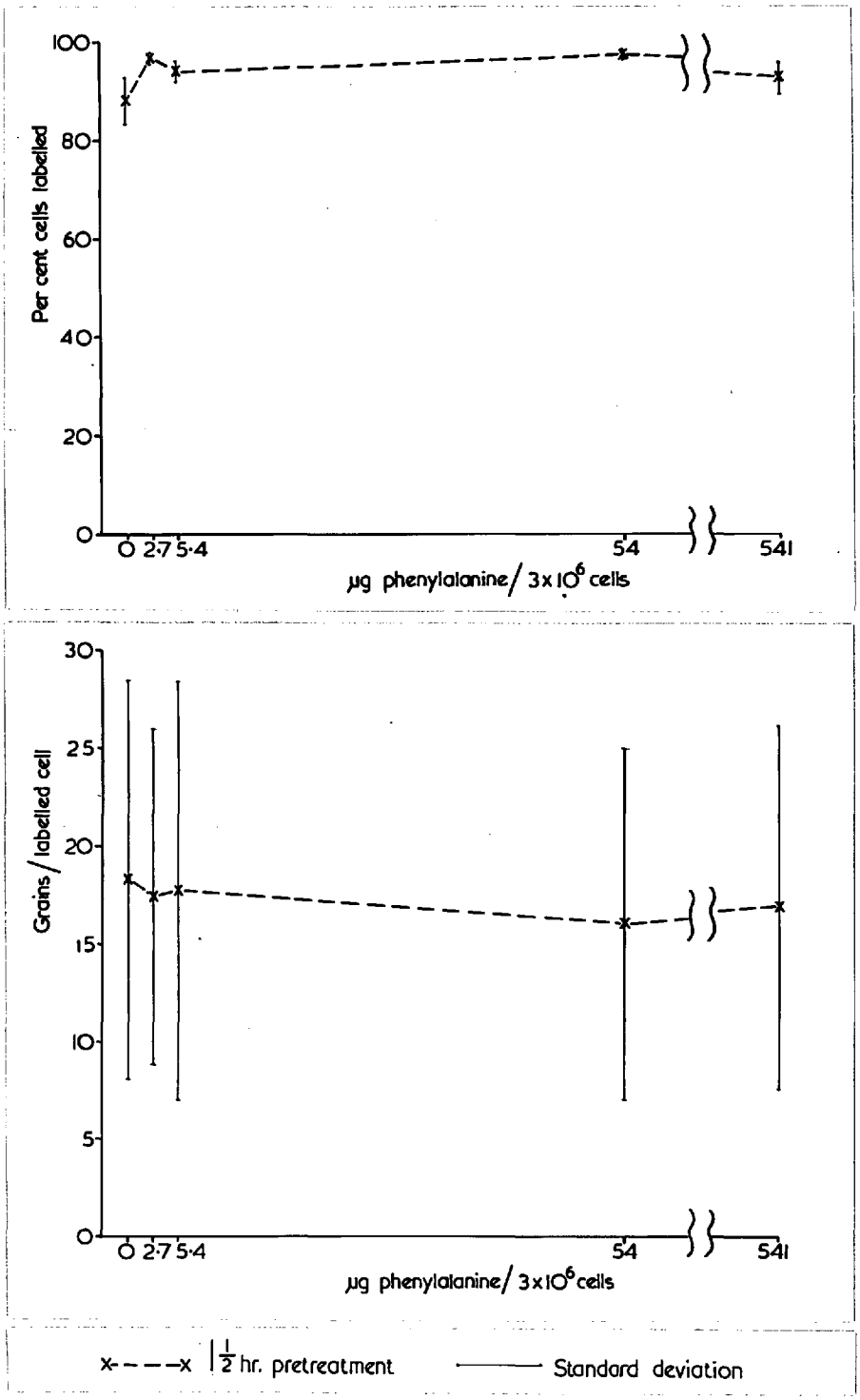


Fig. 32. The effect of phenylalanine on leucine-H^3 uptake by mouse thymocytes <u>in vitro</u>.

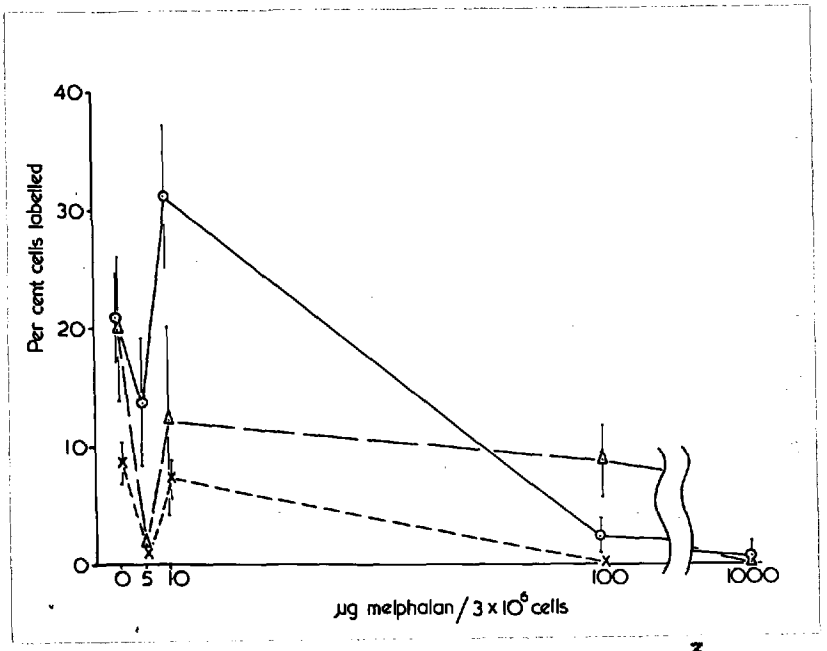


Fig. 33. The effect of melphalan on leucine-H^3 uptake by mouse thymocytes <u>in vitro</u>.

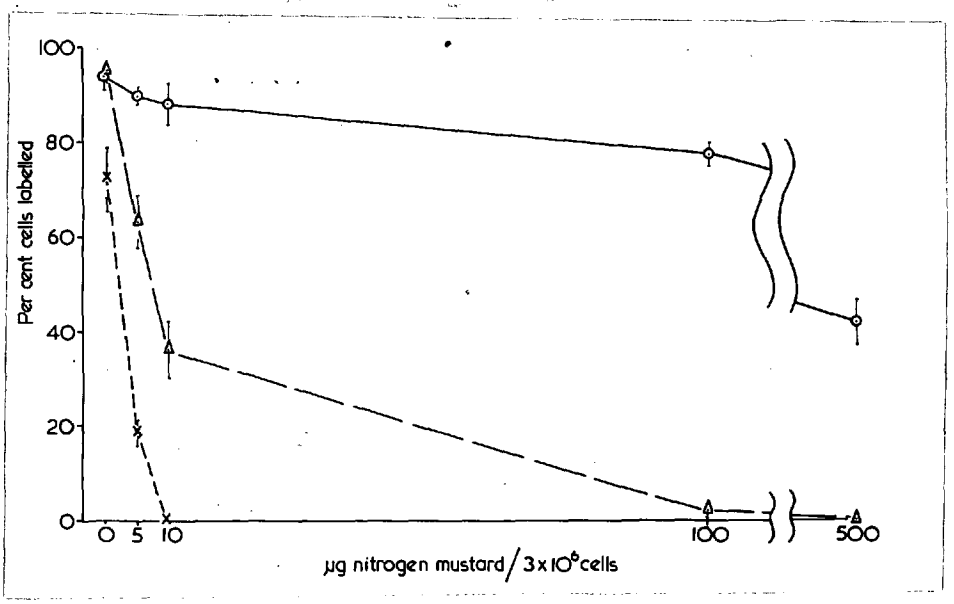
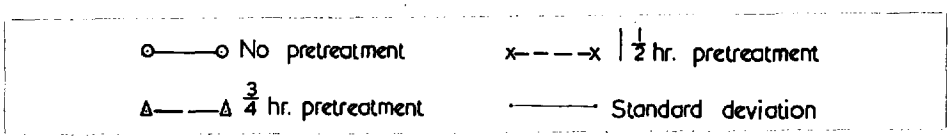


Fig. 34. The effect of nitrogen mustard on leucine-H^3 uptake by mouse thymocytes <u>in vitro</u>.



The thymus from a 23.5 g. female A2G mouse was teased into a cell suspension and adjusted to contain 3×10^6 cells/ml. Leucine-H^3 uptake by the cells in a 43 min. incorporation period was determined after 0, $\frac{3}{4}$ and $1\frac{1}{2}$ hr. pretreatment with melphalan or melphalan diluent. Inhibition of leucine-H^3 uptake did occur, with 1000 μg. and no pretreatment reducing the proportion of labelled cells from the control value of 21 per cent to 0.6 per cent (Fig. 33 and Appendix Table 30). Since $1\frac{1}{2}$ hr. pretreatment with the diluent reduced labelling to only 8.6 per cent cells labelled, the extent of damage after pretreatment due to drug rather than to diluent is difficult to assess.

The expected mode of action of melphalan has been discussed in the Introduction (page 29) and it has been pointed out that the cytotoxicity of this drug is generally considered to be due to the alkylating moiety of the molecule and that no significant antimetabolite effect takes place.

These preliminary experiments indicate that part of the cytotoxic action of melphalan may be due to its phenylalanine content since equivalent amounts of phenylalanine do interfere with tyrosine-H^3 uptake. Since uptake of leucine-H^3 is reduced by melphalan but not by phenylalanine, and since thymidine-H^3 and uridine-H^3 uptake is affected by melphalan, the mustard part of the molecule appears responsible for much of the cytotoxicity of this drug. It is possible that the l-phenylalanine part of the drug aids transport of the drug

into and within the cell. In conclusion, melphalan may owe part of its anti-tumour activity against melanomata to interference with the tyrosine ⟶ melanin pathway. It is also possible that melphalan is incorporated into tumour cells because of its phenylalanine content and that, once there, the mustard part of the molecule causes irreversible damage.

Effect of Nitrogen Mustard on Uptake of Leucine-H^3

Nitrogen mustard inhibits uptake of tyrosine-H^3 at concentrations only slightly higher than those needed to inhibit uptake of thymidine-H^3 and uridine-H^3. To determine whether nitrogen mustard would affect the uptake of a second amino acid in the same way as it affects the uptake of tyrosine-H^3, the experiment was repeated using inhibition of leucine-H^3 uptake as the indication of cell damage.

The thymus from a 15 g. female A2G mouse was made into a cell suspension containing 3×10^6 cells/ml. nutrient medium. The ability of the cells to incorporate leucine-H^3 (10 μc final concentration) in a 30 min. incubation period was assessed after 0, ¾ or 1½ hr. pretreatment with nitrogen mustard. The results were very similar to those for tyrosine-H^3 uptake, with negligible labelling detectable in cells pretreated for 1½ hr. with 10 μg. nitrogen mustard (Fig. 34, Appendix Table 31). It was noticed that, whereas pretreating 3×10^6 cells with 10 μg. nitrogen mustard for ¾ hr. prevented detectable uptake of thymidine-H^3, 36 per cent of the

cells could be labelled with leucine-H^3 and 35 per cent of the cells could be labelled with tyrosine-H^3 after this treatment. This could have been due to preferential damage to cells in the S phase at the time of contact with the drug. Although thymidine-H^3 uptake would be prevented, cells not in the S phase during the incubation time with nitrogen mustard would be capable of incorporating amino acids. The possible preferential damage to S phase cells occurring with certain drug concentrations was investigated further.

Preferential Damage to S Phase Cells

Walker and Helleiner (1963) found that interphase cells are more sensitive to nitrogen mustard and sulphur mustard during the period of DNA synthesis (S phase) than in the other interphase stages. The cells they used (Earle's 'L' cells in vitro) were first partially synchronized with 5-fluoro-2'-deoxyuridine and thymidine, and this treatment may have affected the subsequent response of the cells to nitrogen mustard. However, the effects of nitrogen mustard on the incorporation of thymidine-H^3 and amino acids by mouse thymocytes appear to fit their hypothesis, in that $\frac{3}{4}$ hr. pretreatment with nitrogen mustard at a concentration of 10 µg./3 x 10^6 cells prevented thymidine-H^3 uptake although 35 per cent of the cells could be labelled with tyrosine-H^3 and 36 per cent with leucine-H^3 after this treatment. Similar results have been found with tretamine. It has been shown that only cells in the S phase are capable of incorporating thymidine-H^3 and that very few small thymocytes become

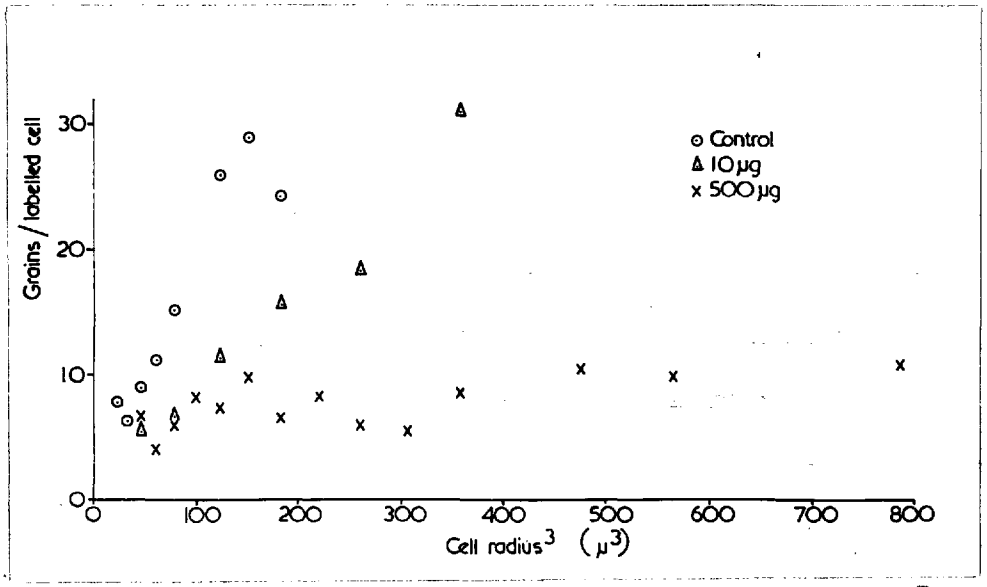


Fig. 35. The effect of nitrogen mustard on tyrosine-H^3 uptake by mouse thymocytes of different sizes.

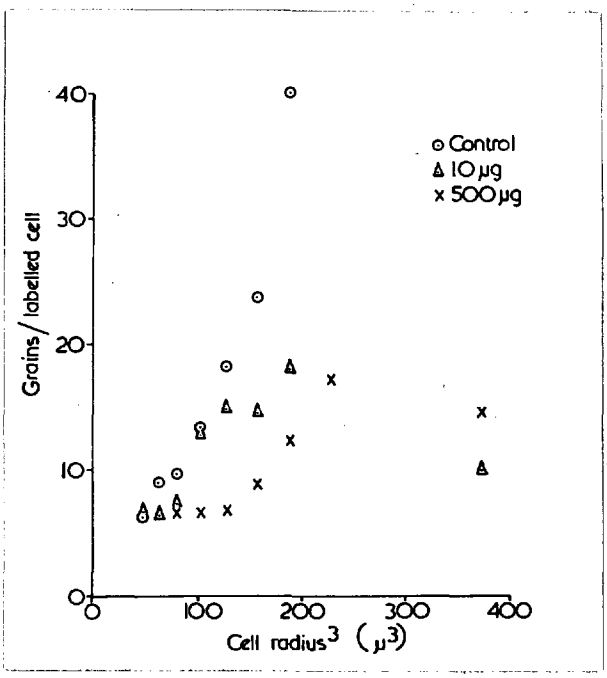


Fig. 36. The effect of nitrogen mustard on leucine-H^3 uptake by mouse thymocytes of different sizes.

labelled after a ½ hr. incubation period with thymidine-H^3. If a selective action against cells in the S phase were taking place in the mouse thymocytes, then small thymocytes would be expected to incorporate tyrosine-H^3 and leucine-H^3 to the same extent as untreated controls whereas amino acid uptake by some of the large cells would be inhibited or reduced. Slides showing incorporation of tyrosine-H^3 and leucine-H^3 by mouse thymocytes treated with 500 µg. nitrogen mustard without pretreatment, with 10 µg. with ¾ hr. pretreatment or with diluent only without pretreatment were examined. The average grain count for each cell size was determined from these smears.

From the tyrosine-H^3 series, it was found that pretreating the cells with 10 µg. nitrogen mustard for ¾ hr. reduced the mean number of grains for cells of all sizes to about the same extent (Fig. 35, Appendix Table 32). Treating the cells with 500 µg. immediately before the addition of tyrosine-H^3 had a more drastic effect on the mean number of grains for each cell size and large cells were covered by the same number of grains as smaller cells. There is no indication of a preferential effect on tyrosine-H^3 uptake by large cells. One complicating factor is that since 500 µg. nitrogen mustard causes an increase in cell size it is difficult to tell which cells in the drug-treated population correspond to the large cells in the untreated population. It is possible that what appeared to be large cells in the drug-treated population originated as small thymocytes and that

the original large cells might have been damaged to such an extent that they were undetectable because of lysis.

Cell sizes were similarly measured on smears of thymocytes incubated with nitrogen mustard and labelled with leucine-H^3 during a 43 min. incorporation period. As with tyrosine-H^3 uptake, the extent of leucine-H^3 uptake per cell without pretreatment and in the presence of 0.1 ml. water was proportional to the cell size. Pretreating the mouse thymocytes with 10 μg. nitrogen mustard for ¾ hr., however, resulted in the larger cells taking up less label than in the control cell sample (Fig. 36, Appendix Table 33). This was more apparent when the cells were incubated with 500 μg. nitrogen mustard without pretreatment. Allowing for the increase in cell size, cells originally 5 μ (11.5 μ after exposure to nitrogen mustard) or less incorporated on average the same quantity of leucine-H^3 per cell as the corresponding control cells, the uptake being proportional to the cell size. Cells greater than 11.5 μ (probably about 9 μ originally) incorporated the same quantity of leucine-H^3 whatever the size.

Thus, there is some indication of preferential drug effect on the large cells in the figures for leucine-H^3 uptake but not for tyrosine-H^3 uptake. No explanation for these contradictory results can be offered except that the measurements for the two series were made by two different observers and that, since 500 μg. nitrogen mustard without pretreatment had a more pronounced effect

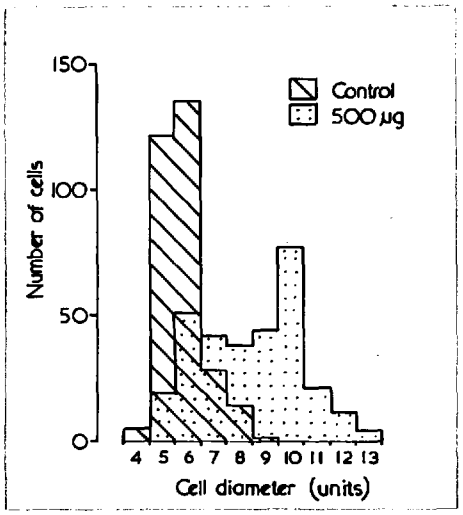


Fig. 37

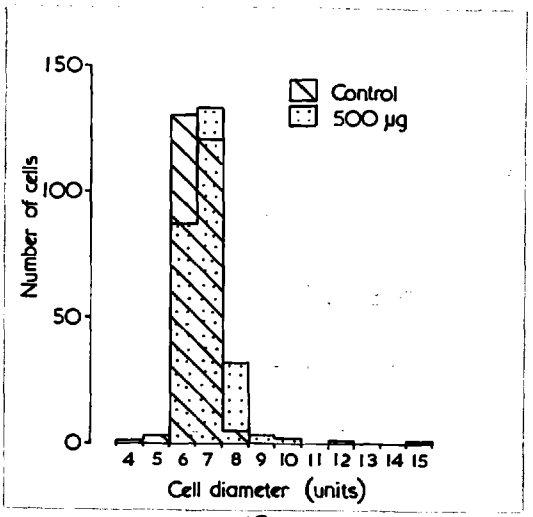


Fig. 38

Fig. 37. The effect of nitrogen mustard on thymocyte size (tyrosine-H^3 experiment). 1 unit = 1.42 µ.

Fig. 38. The effect of nitrogen mustard on thymocyte size (leucine-H^3 experiment). 1 unit = 1.44 µ.

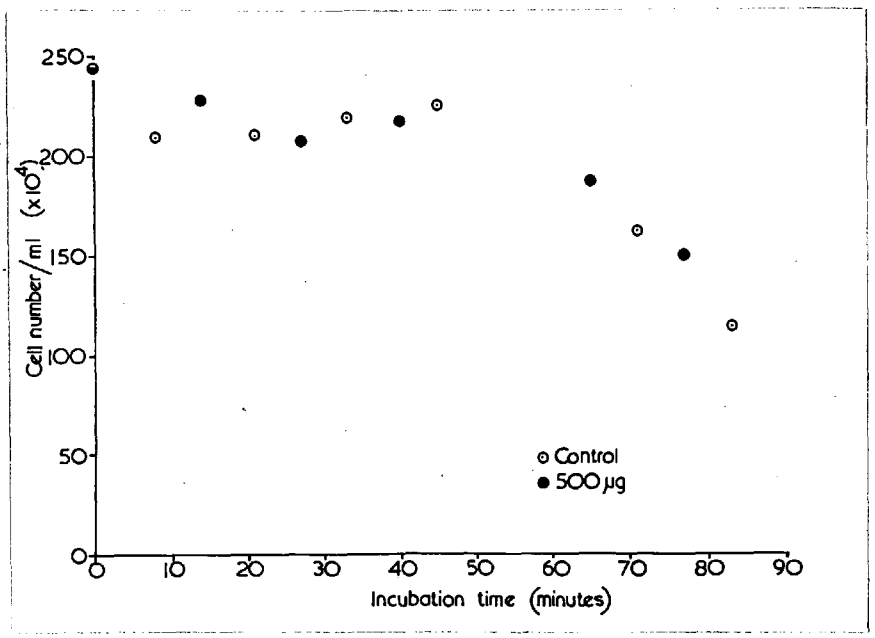


Fig. 39. The effect of nitrogen mustard on the concentration of mouse thymocytes <u>in vitro</u>.

on cell size in the tyrosine-H^3 series (Fig. 37, Appendix Table 32) than in the leucine-H^3 series (Fig. 38, Appendix Table 33), there may have been slight differences in the sensitivities of the two cell populations or in the drug concentrations used.

The effect of nitrogen mustard on cell concentration and cell size was investigated to determine whether the very large cells after exposure to drug were originally small or large cells, and whether any cells had lysed.

Effect of Nitrogen Mustard on Cell Concentration

It will be shown in the next section that the very large cells resulting from exposure of mouse thymocytes to nitrogen mustard originate from medium and large thymocytes. Some of these large thymocytes may have increased in size to such an extent that the cells lysed. However, a single experiment to show the effect of 500 μg. nitrogen mustard on the cell concentration in 1 ml. aliquots of cell suspension indicated that there was no difference between the decrease in cell number in the presence of nitrogen mustard and the decrease in cell number in the diluent controls (Fig. 39 and Appendix Table 34). It is concluded that 500 μg. nitrogen mustard does not affect the cell concentration to a greater extent than the control conditions for at least $1\frac{1}{2}$ hr. after the beginning of incubation.

Effect of Nitrogen Mustard on Thymocytes Previously Labelled with Thymidine-H^3

Nitrogen mustard causes an increase in cell size (Levis, Spanio

TABLE IX. THE EFFECT OF NITROGEN MUSTARD ON LABELLING OF MOUSE THYMOCYTES PREVIOUSLY INCUBATED WITH THYMIDINE-H^3

Incubation time after addition of drug (min.)	Drug concentration (µg. / 3 x 10^6 cells)		
	0	10	100
	Percentage of cells labelled (± standard deviation)		
30	9.3 ± 3.5	7.4 ± 2.3	9.8 ± 3.9
60	12.9 ± 5.0	10.6 ± 3.2	10.4 ± 3.6
90	12.6 ± 3.3	11.7 ± 4.9	11.2 ± 3.6

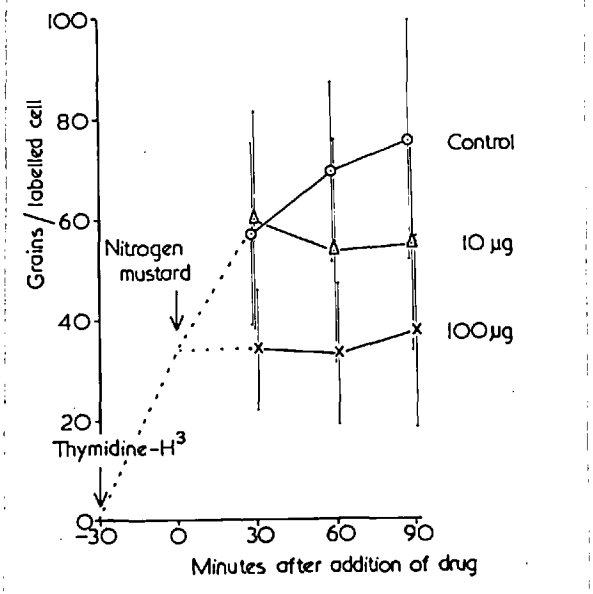


Fig. 40. The effect of nitrogen mustard on labelling of mouse thymocytes previously incubated with thymidine-H^3

and de Nadai, 1963; Fig. 37, 38). To determine whether the very large cells found after exposure of thymocytes to nitrogen mustard originate as small or large cells, and also to determine whether cells incorporating thymidine-H^3 at the time of contact with nitrogen mustard will continue to do so, the effect of nitrogen mustard on cells previously incubated with thymidine-H^3 was studied.

A cell suspension containing 3×10^6 cells/ml. was prepared from the thymus of a 24 g. female A2G mouse. Thymidine (0.1 ml.) was added to 1 ml. aliquots of cell suspension to give a final concentration of 0.1 μc thymidine-H^3/3×10^6 cells. All the tubes were incubated at 37°C for ½ hr., then 0.1 ml. of freshly prepared nitrogen mustard (at room temperature) was added to each of three of the tubes to give a final drug concentration of 10 μg./3×10^6 cells and to a second set of three tubes to give a concentration of 100 μg./3×10^6 cells. Water (0.1 ml.) was added to the three control tubes. Incubation at 37°C was continued in the presence of nitrogen mustard or water for a further 30, 60 or 90 min., after which time the cells were washed and smeared as usual.

There was little change in the proportion of cells labelled with thymidine-H^3 whether the cells were subsequently incubated with nitrogen mustard or diluent (Table IX). In the smears from the diluent controls, the cells showed a mean grain count/labelled cell which increased with time (Fig. 40, Appendix Table 35). This

increased uptake was not linear, probably because the thymidine-H^3 concentration had been reduced and the cells had been cooled slightly when the water was added. The addition of 100 μg. nitrogen mustard caused an immediate stop in the increase in the mean number of grains with time. The addition of 10 μg. nitrogen mustard allowed an increase in the mean grain number per labelled cell for about 30 min., but after this time the mean grain count remained unchanged for the next 60 min. This agrees with the thymidine-H^3 results in Appendix Table 14, which shows that mouse thymocytes pretreated with 10 μg. nitrogen mustard for $\frac{3}{4}$ hr. failed to take up thymidine-H^3 in a subsequent 35 min. incorporation period whereas this drug concentration had a negligible effect without pretreatment. 100 μg. nitrogen mustard appears to cause an immediate block in thymidine-H^3 uptake while 10 μg. allows uptake to continue for a short time because there is less chance of a drug molecule reaching a target. Since there is some increase in the percentage of cells labelled after the addition of 100 μg. nitrogen mustard, some of the labelled cells must have entered the S phase after the drug addition and these cells would have had low grain counts. Although the mean grain count/labelled cell is the same 90 min. after drug addition as 30 min. after, some increase in grain number over those cells already synthesising DNA at the time of drug contact must have occurred to compensate for these low values. Slight uptake of thymidine-H^3, therefore, probably occurred in the presence of 100 μg. nitrogen mustard.

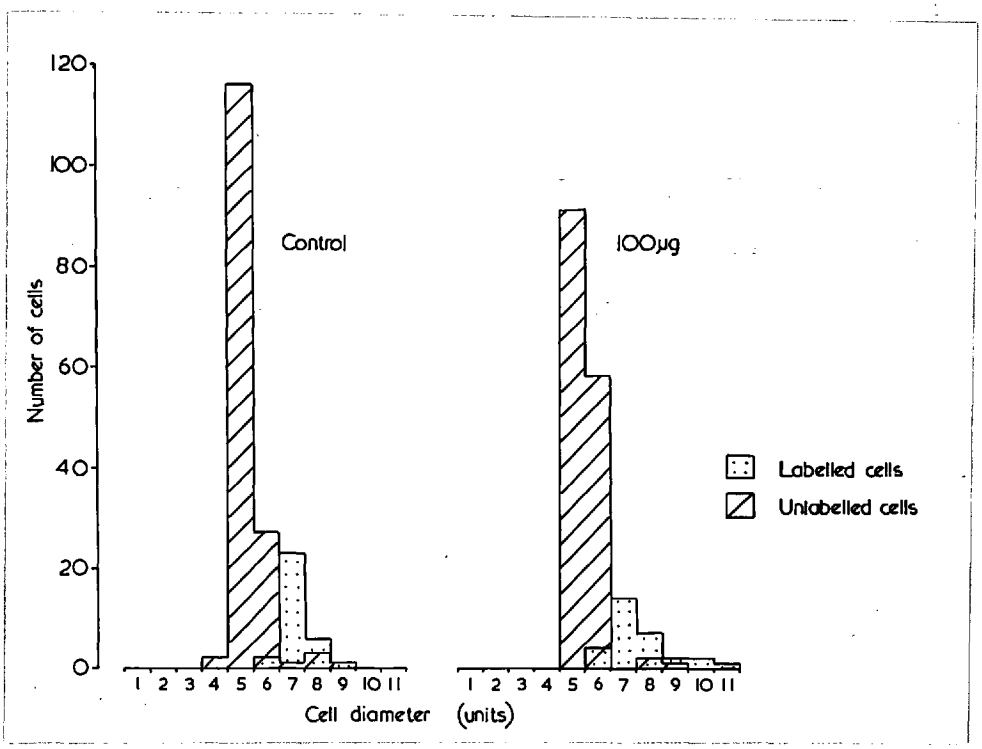


Fig. 41. The effect of nitrogen mustard on the cell size of thymocytes previously labelled with thymidine-H^3 and of unlabelled thymocytes. 1 unit = 1.43 µ.

The diameters of labelled and unlabelled cells were measured from smears of cells incubated with thymidine-H^3 for $\frac{1}{2}$ hr. and then exposed to 100 µg. nitrogen mustard or diluent for a further 90 min. There were more cells with a relatively large diameter after exposure to nitrogen mustard (Fig. 41, Appendix Table 36). Since as high a concentration of nitrogen mustard as 500 µg has the same effect on cell concentration as the diluent (page 130) this is presumably not due to loss of small cells but is due to an increase in the size of individual cells.

The labelled cells found after exposure to nitrogen mustard or water are those which had been in the S phase during the incubation time with thymidine-H^3. Since 100 µg. nitrogen mustard stops most of the labelling, labelled cells after exposure to nitrogen mustard probably originated as large or medium thymocytes. Small thymocytes from the original cell sample would not become labelled. Since an increase in the size of both labelled and unlabelled cells can be seen, nitrogen mustard affects the size of both potentially-dividing and non-dividing cells. In other words, there is no preferential effect on the size of the S phase cells. But, since the very large cells resulting from exposure to nitrogen mustard are labelled, the giant cells in the tyrosine-H^3 and leucine-H^3 experiments have originated from medium and large thymocytes and not from small thymocytes.

DISCUSSION

The object of an *in vitro* sensitivity test for an individual human tumour is to obtain results most likely to predict the response of that tumour *in situ* to cancer chemotherapeutic agents. Using mouse thymocytes as a model test system and inhibition of thymidine-H^3 uptake as an indication of cell damage, it was found that the relative effectiveness of selected alkylating agents in damaging the cells depended on how long the cells were pretreated with drug before the ability to incorporate thymidine-H^3 was assessed. Different drugs appeared to be the most effective in reducing thymidine-H^3 uptake for each of the three time schedules used. Bickis, Henderson and Quastel (1965) described an *in vitro* sensitivity test which was based on a single pretreatment time of 30 minutes. They obtained some correlation between the predictions of the *in vitro* test and the response of individual patients to the drugs administered. It is quite possible, however, that their results would have differed if they had selected another pretreatment time and the correlation between the *in vitro* results and the clinical progress of the patients might have been stronger (or weaker). Since the results of an *in vitro* sensitivity test are influenced by the extent of the pretreatment effects of the cytotoxic agents, the pretreatment effects in mouse thymocytes were thought worthy of further investigation.

Experimental results have been produced which demonstrate

that the pretreatment effect produced by nitrogen mustard is not the result of a lag period before the damage becomes detectable. It is suggested that this drug acts continuously on the cells and produces cumulative damage. The fact that alkylating radicals were still detectable 1½ hours after 10 μg. nitrogen mustard was added to cells in nutrient medium at 37°C suggests that active drug does remain in the medium throughout the longest pretreatment time used. Alkylating radicals can also be detected for chlorambucil (initially 100 μg.) after this time, although chlorambucil appears to be no more effective in inhibiting thymidine-H^3 uptake after 1½ hours pretreatment than without pretreatment. The lack of an enhanced drug effect after pretreatment with chlorambucil cannot be due to the exhaustion of the alkylating ability of the drug. The fact that chlorambucil reacts by a unimolecular mechanism while nitrogen mustard reacts by a bimolecular mechanism is unlikely to be the explanation for the extremes of pretreatment effect shown by these two drugs since melphalan also reacts by a unimolecular mechanism but produces a pronounced pretreatment effect. The suggestion is made that chlorambucil may be acting in a different manner from nitrogen mustard. It may be significant that nitrogen mustard and chlorambucil carry opposite charges when in solution, the ethyleneimonium ion resulting from cyclization of nitrogen mustard acting as a cation and chlorambucil acting as an anion. Melphalan carries both a cationic and an anionic group and the compounds with an inter-

mediate pretreatment effect (tretamine and thioTEPA) are neutral in solution.

Even if it can be accepted that the damage produced on the thymocytes by nitrogen mustard is continuous and cumulative, this fact gives no indication of the mode of action of this drug. It was suggested in the Introduction (page 25) that nitrogen mustard may act by producing a block in the G2 phase of the cell cycle (Layde and Baserga, 1964; Gelfant, 1963). Would a pretreatment effect be expected if nitrogen mustard did act on the cell cycle in this manner? According to this hypothesis, cells in the latter part of the G2 phase at the time of contact with the drug would divide, pass through the G1 phase and enter the S phase. If these cells were in part of the S phase during the incubation time with thymidine-H^3, they would appear labelled on an autoradiograph. Once these cells had passed through S, however, the block in the G2 phase would prevent them entering a further cell cycle. The longer the cells were in contact with the nitrogen mustard, the greater the number of mitoses prevented, the fewer the number of cells entering the S phase and therefore the lower the percentage of tritium-labelled cells. Thus, a pretreatment effect would result from a G2 block. But, to prevent labelling with thymidine-H^3 solely by a block in the G2 phase, a minimum pretreatment time would be needed which was equal to the duration of any G2 phase remaining after the block, plus the duration of mitosis, plus G1,

plus S. Metcalf and Wiadrowski (1966) found that the length of the S phase alone of large and medium mouse thymocytes was 5.5 hours and the cell cycle of small thymocytes considerably longer. Since, in the present experiments, inhibition of detectable uptake of thymidine-H^3 occurred after only $1\frac{1}{2}$ hours pretreatment with tretamine, $\frac{3}{4}$ hour pretreatment with nitrogen mustard and melphalan and without pretreatment with chlorambucil and thioTEPA, a block in the cell cycle cannot be the sole mode of action of these drugs under these experimental conditions. In the same way a single block in any other part of the cell cycle (such as the elongation of the G1 phase due to thioTEPA found by Fujita, Horii, Tanimura and Nishimura, 1964) can also be excluded as the sole mode of action of these drugs. These conclusions are supported by the fact that inhibition of uridine-H^3 and tritiated amino acids occurs in mouse thymocytes treated with approximately the same concentrations needed to inhibit thymidine-H^3 uptake. A block in the cell cycle would not be likely to interfere with other metabolic processes in the cells.

Alkylation of DNA components followed by the incorporation of the analogues into DNA to produce an antimetabolite effect has been suggested by Lorkiewicz and Szybalski (1961) as the mode of action of tretamine, though their results have been questioned by Lawley and Brookes (1963). However, since exogenous thymidine was supplied in the mouse thymocyte experiments, alkylation of this nucleoside may have occurred in the medium and may have prevented the

incorporation of the label into the cells. The cells may have been relatively undamaged by drug but unlabelled with thymidine-H^3 because the thymidine-H^3 became bound to drug in the medium rather than being incorporated into the cell for DNA systhesis. The experiment described on page 91 in which the supernatent fluid over drug-treated cells was removed after centrifugation and replaced by fresh medium and thymidine-H^3, contradicts the idea of such an interaction between nitrogen mustard and thymidine-H^3. The thymidine-H^3 did not come into direct contact with the nitrogen mustard in the medium and yet the drug still prevented uptake of the labelled metabolite at the appropriate concentrations and pretreatment times. In the experiment (page 79) in which the nitrogen mustard was not removed from the medium before the thymidine-H^3 was added, labelling was prevented in cells pretreated with 10 µg./ml. nutrient medium for ¾ hour. When the drug was removed before the addition of thymidine-H^3, however, a pretreatment period of 1½ hours with 10µg. nitrogen mustard/ml. nutrient medium prevented detectable labelling whereas a pretreatment period of ¾ hour with this drug concentration did not. The slight difference can probably be explained by the fact that in the former experiment the drug was present during the 35 minutes incorporation period for the thymidine-H^3 as well as during the pretreatment period and the cells were therefore being pretreated for a period of ¾ hour plus 35 minutes. It can be concluded that nitrogen mustard has the same effect on thymidine-H^3

uptake by mouse thymocytes whether or not the drug comes into direct contact with the labelled metabolite, and that, with this drug at least, the pretreatment effect is not due to an interaction between the drug and the thymidine-H^3.

The controversy over whether the alkylating agents damage cells by selective action on the DNA has been mentioned in the Introduction (page 20-1). Interference with DNA synthesis by drug concentrations which have no effect on RNA synthesis has been demonstrated from the time of Bodenstein and Kondritzer (1948) and the cytological effects produced by the alkylating agents involve mainly the DNA (for example, see Auerbach and Robson, 1946; Koller, 1958). In spite of this, there are reports of other metabolic pathways being affected by the alkylating agents (see the review by Wheeler, 1962), though when this occurs the concentration of drug tends to be relatively high (Table I, page 22). With mouse thymocytes, it was found that nitrogen mustard inhibited uptake of tyrosine-H^3 and leucine-H^3 under slightly more drastic conditions ($1\frac{1}{2}$ hours pretreatment with 10 µg.) than those needed to reduce the uptake of thymidine-H^3 and uridine-H^3 to negligible proportions ($1\frac{1}{2}$ hours pretreatment with 5 µg.). This indicates that DNA synthesis is not the sole metabolic process affected by these dose ranges of nitrogen mustard under these experimental conditions. Tretamine acted in a similar manner to nitrogen mustard in that 1000 µg. tretamine prevented thymidine-H^3 uptake after $1\frac{1}{2}$ hours

pretreatment and reduced tyrosine-H^3 uptake. The effect of thioTEPA on tyrosine-H^3 uptake was similar to its effect on thymidine-H^3 uptake. The toxicity of the chlorambucil and melphalan diluents obscured the effects of these drugs on the uptake of uridine-H^3 and the tritiated amino acids after pretreatment, though without pretreatment there was some reduction in the uptake of uridine-H^3 in the presence of drug concentrations which prevented uptake of thymidine-H^3. The uptake of leucine-H^3 by mouse thymocytes was reduced to negligible proportions without pretreatment by a concentration of melphalan (1000μg.) which had no effect on the uptake of thymidine-H^3 or tyrosine-H^3 by mouse thymocytes without pretreatment. There is some slight evidence that part of the action of melphalan against melanomata may be due to the drug's phenylalanine content producing an antimetabolite effect. Since, in general, the drug concentrations which inhibited the uptake of thymidine-H^3 by mouse thymocytes also reduced the uptake of other metabolites, the alkylating agents cannot be acting selectively on DNA synthesis, under the experimental conditions used.

Mannitol myleran had no detectable effect on the mouse thymocytes, even when these were pretreated with 2 mg./3 x 10^6 cells (4.9 mM) for 1½ hours before a labelled metabolite was added. Even after this treatment, the percentage of cells labelled and the mean number of grains over labelled cells was the same as in the diluent controls, whether thymidine-H^3, uridine-H^3 or tyrosine-H^3 was used

as the labelled metabolite. This agrees with the results of Onesti and Woodliff (1963) who found that as high a concentration as 33.8 mg. mannitol myleran/ml. had no effect on oxygen uptake by chronic granulocytic leukaemia cells in vitro. Similarly, Reid (1962) reported that 0.5 mg./ml. had no effect on the in vitro synthesis of uridine nucleotides and glycogen by rat liver. On the other hand, Kellner and Németh (1959) found that 1 mg. mannitol myleran/ml. had a marked in vitro effect after 24 hours on fibroblasts of chick embryo hearts, fresh Crocker sarcoma and HeLa cells, and 2 mg./ml. caused cell destruction. As low a concentration as 2.5 to 250 µg./ml. was found by Easty and Wylie (1963) to have a cytotoxic effect on glioma cells. There seems to be a wide range of sensitivity to mannitol myleran from tissue to tissue and it appears that mouse thymocytes are relatively resistant to this drug after this incubation time. It may be that mannitol myleran acts so slowly that there was not sufficient time for any damage to become detectable.

Since nitrogen mustard and tretamine can cause inhibition of thymidine-H^3 uptake under conditions which have only a partial effect on tyrosine-H^3 uptake, it was thought that there might be preferential damage to cells in the S phase at the time of contact with these two alkylating agents (Walker and Helleiner, 1963). The results are contradictory in that leucine-H^3 uptake in the presence of certain concentrations of nitrogen mustard is reduced in large cells only (those cells likely to be in the S phase) while tyrosine-H^3

uptake under the same conditions is reduced in cells of all sizes. However, the figures for the distribution of cell size after drug-treatment (page 128) indicate that there is a more pronounced effect on cell size in the tyrosine-H^3 experiment than in the leucine-H^3 experiment. This could be due to slight differences in the sensitivities of the two cell populations or in the drug concentrations used. The drug in the tyrosine-H^3 experiment had a more drastic effect on cell size and may, therefore, have had a more drastic effect on cell metabolism which might have masked any preferential damage to S phase cells. Since amino acid uptake can be prevented in the presence of these alkylating agents after short pretreatment times, attack on S phase cells cannot be the whole explanation of the pretreatment effect. Small thymocytes (which rarely divide) would remain undamaged for a very long time if damage occurred solely during the S phase. The S phase cells may, however, be relatively more susceptible to damage.

From these considerations, it can be concluded that these drugs do not have a selective action on DNA synthesis or on S phase cells and that they do not produce a block in the cell cycle of the mouse thymocytes. The most likely explanation for the results obtained is that these cytotoxic agents interfere with several metabolic processes and cause non-specific cell death. The extent of drug reaction within the cells may depend on the length of time the drug is allowed to act on the cells, and the extent of the pretreatment effect may depend on

the rate of reaction of the drug used (though chlorambucil may be acting in a different manner from the other alkylating agents).

Practical implications. Relatively high drug concentrations were used *in vitro* to allow damage to become detectable within a short time. But, with the alkylating agents used for cancer chemotherapy at the present time, the dose levels that can be attained *in vivo* are comparatively low to minimize damage to normal tissue such as bone marrow and intestinal mucosa. The drugs may act in a different manner at these lower doses, and the relative effectiveness of several drugs *in vivo* may not be reflected by the relative effectiveness of the same drugs under the proposed *in vitro* conditions. The ultimate aim of cancer chemotherapy is to kill 100 per cent of the tumour cells (Skipper, 1964; Goldenberg and Alexander, 1965) and the best drug for clinical use would in theory be the one with the greatest ability to kill all the cells from a sample of the individual tumour. An *in vitro* sensitivity test of the type proposed here may well be suitable even though a general drug effect on several metabolic pathways is being measured.

It has been found that the relative effectiveness of the selected drugs *in vitro* depends on the incubation schedule followed and it is not known which, if any, of these time schedules would be the best to use. If the relative effectiveness of these drugs at therapeutic doses could be determined *in vivo* in laboratory animals, the incubation procedure *in vitro* could probably be suitably modified to produce

results most likely to predict *in vivo* results. A further complication is the fact that the length of the cell cycle of the majority of human tumours is longer than that of mouse thymocytes and, therefore, relatively few cells would take up thymidine-H^3 in a short incubation period. However, since the effects of the alkylating agents on uptake of the labelled amino acids were similar to the effects of these agents on the uptake of thymidine-H^3, it may be possible to use the uptake of labelled amino acids as the indication of cell metabolism in samples of individual tumours.

The mode of action of the drugs to be used in an *in vitro* sensitivity test would have to be known before the results could be interpreted. For example, a drug such as cyclophosphamide, which is relatively inactive *in vitro* and which is converted to an active form *in vivo* (Foye, Chapman, Willett and Adams, 1960), is unlikely to affect the uptake of labelled metabolites *in vitro* although the tissue *in situ* may be sensitive to that particular drug. As a further example, the increased uptake of thymidine-H^3 by individual cells in the presence of methotrexate (page 109) does not reflect stimulated DNA synthesis but is due to a blockage in the uridine to thymidine pathway (O'Brien, 1962). Comparison of decreased thymidine-H^3 uptake in the presence of one drug (for example an alkylating agent) and increased thymidine-H^3 uptake in the presence of an antifolate would be difficult to evaluate at the quantitative level. Prior experience with these drugs under these

in vitro conditions would probably help. Moreover, the test is intended only as an aid to drug selection and factors such as the relative drug toxicities and the state of the patient would also need to be considered.

A further problem is the preparation of cell suspensions from solid tumours. The use of uniform slices of solid tissue (as used by Wolberg and Curreri, 1961; Wolberg and Brown, 1962) has been rejected because labelling with thymidine-H^3 is confined to the peripheral cell layers. This difficulty was also found by McDonald *et al.* (1963). Moreover, the extent of labelling with thymidine-H^3 has been shown to depend on the thymocyte concentration and the cell concentration is easier to control in a suspension than in supposedly uniform slices. Some work on the preparation of cell suspensions from solid mammalian tissues has been attempted (by the author) and it appears that the method of preparation will need to be modified for each individual tumour. A method sufficiently drastic to produce single cells tends to prevent the uptake of labelled metabolites by the cells within a short incubation period. Other workers have prepared cell suspensions from solid tumours but usually with the aim of establishing the cells in tissue culture. When the cells are being incubated for several days, a low initial cell viability may be sufficient to produce growth, but the cells growing may represent only a small proportion of the cell types originally present in the solid tumour. Moore, Mount, Tara and Schwartz (1963) state that so

far they have been unable to establish suspension cultures of human cells directly from effusions or biopsy specimens.

There is, clearly, a need for an *in vitro* test which can predetermine the sensitivity of an individual patient's tumour to cancer chemotherapeutic agents. These initial studies indicate that mammalian cells (mouse thymocytes *in vitro*) are not equally sensitive to all cytotoxic agents but rather that these cells are more sensitive to some of the drugs than to others. By measuring, on autoradiographs, the uptake of labelled metabolites by drug-treated cells, the extent of interference with various metabolic pathways and the proportion of damaged cells can be determined. In spite of the fact that the response of mammalian tissue *in vitro* to cytotoxic agents can be measured at the cellular level and in spite of the fact that disrupted metabolic pathways can be detected relatively easily, a vast amount of laboratory work needs to be done before the *in vitro* sensitivity test can be used at the clinical level. The major problem is how to prepare cell suspensions from solid tissues without affecting the ability of the cells to incorporate labelled metabolites. There is also the need to determine whether the *in vitro* test can produce results which will predict the response of the tumour *in situ* to cancer chemotherapeutic agents. If these problems could be solved, however, the *in vitro* sensitivity test for individual human tumours would have wide application and would be of great value in the treatment of neoplastic disease.

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LIST OF APPENDIX TABLES

Table		Page
1.	Correlation between cell size and uptake of thymidine-H^3 by mouse thymocytes *in vitro*	167
2.	Correlation between thymocyte size and the grain number over cells labelled *in vitro* with thymidine-H^3	168
3.	The effect of cell concentration on thymidine-H^3 uptake by mouse thymocytes *in vitro*	169
4.	Rate of thymidine-H^3 uptake by mouse thymocytes *in vitro*	170
5.	The effect on labelling of a time lag at 37° C between the preparation of a thymocyte suspension and incubation with thymidine-H^3	171
6.	The uptake of tyrosine-H^3 by mouse thymocytes *in vitro*	172
7.	The uptake of leucine-H^3 by mouse thymocytes *in vitro*	173
8.	Correlation between thymocyte size and the grain number over cells labelled *in vitro* with leucine-H^3	174
9.	Correlation between thymocyte size and the grain number over cells labelled *in vitro* with tyrosine-H^3	175
10.	The effect of nitrogen mustard (diluted in saline) on thymidine-H^3 uptake by mouse thymocytes *in vitro*	176
11.	The effect of mannitol myleran on thymidine-H^3 uptake by mouse thymocytes *in vitro*	177
12.	The effect of chlorambucil on thymidine-H^3 uptake by mouse thymocytes *in vitro*	178
13.	The effect of tretamine on thymidine-H^3 uptake by mouse thymocytes *in vitro*	179

Table | Page

14. The effect of nitrogen mustard (diluted in water) on thymidine-H^3 uptake by mouse thymocytes *in vitro* 180

15. The effect of thioTEPA on thymidine-H^3 uptake by mouse thymocytes *in vitro* 181

16. The effect of melphalan on thymidine-H^3 uptake by mouse thymocytes *in vitro* 182

17. The effect of nitrogen mustard on uridine-H^3 uptake by mouse thymocytes *in vitro* 183

18. The effect of melphalan on uridine-H^3 uptake by mouse thymocytes *in vitro* 184

19. The effect of chlorambucil on uridine-H^3 uptake by mouse thymocytes *in vitro* 185

20. The effect of mannitol myleran on uridine-H^3 uptake by mouse thymocytes *in vitro* 186

21. The effect of methotrexate on thymidine-H^3 uptake by mouse thymocytes *in vitro* 187

22. The effect of methotrexate on uridine-H^3 uptake by mouse thymocytes *in vitro* 188

23. The effect of nitrogen mustard on tyrosine-H^3 uptake by mouse thymocytes *in vitro* 189

24. The effect of thioTEPA on tyrosine-H^3 uptake by mouse thymocytes *in vitro* 190

25. The effect of tretamine on tyrosine-H^3 uptake by mouse thymocytes *in vitro* 191

Table		Page
26.	The effect of mannitol myleran on tyrosine-H^3 uptake by mouse thymocytes *in vitro*	192
27.	The effect of melphalan on the uptake of tyrosine-H^3 (5 μc/ml.) by mouse thymocytes *in vitro*	193
28.	The effect of phenylalanine on the uptake of tyrosine-H^3 (1 μc/ml.) by mouse thymocytes *in vitro*	194
29.	The effect of phenylalanine on leucine-H^3 uptake by mouse thymocytes *in vitro*	195
30.	The effect of melphalan on leucine-H^3 uptake by mouse thymocytes *in vitro*	196
31.	The effect of nitrogen mustard on leucine-H^3 uptake by mouse thymocytes *in vitro*	197
32.	The effect of nitrogen mustard on tyrosine-H^3 uptake by mouse thymocytes of different sizes	198
33.	The effect of nitrogen mustard on leucine-H^3 uptake by mouse thymocytes of different sizes	200
34.	The effect of nitrogen mustard (500 μg./ml) on the concentration of mouse thymocytes *in vitro*	202
35.	The effect of nitrogen mustard on labelling of mouse thymocytes previously incubated with thymidine-H^3	203
36.	The effect of nitrogen mustard on the cell size of thymocytes previously labelled with thymidine-H^3 and of unlabelled thymocytes	204

APPENDIX TABLE 1

Correlation between cell size and uptake of thymidine-H^3 by mouse thymocytes *in vitro*. Equal numbers of labelled and unlabelled cells were examined on an autoradiograph of thymocytes incubated at 37° C with 0.1 µc thymidine-H^3 / 3.6×10^6 cells for ½ hr.

Cell diameter		No. of unlabelled cells	No. of labelled cells
Units	µ		
4	4.62	1	0
5	5.78	3	0
6	6.94	49	0
7	8.09	65	0
8	9.25	184	45
9	10.41	18	67
10	11.56	8	62
11	12.72	4	62
12	13.87	4	94
13	15.03	0	4
14	16.19	0	2
Total		336	336

APPENDIX TABLE 2

Correlation between thymocyte size and the grain number over cells labelled *in vitro* with thymidine-H^3

Cell diameter		Radius^3 (μ^3)	No. of cells Total 260	Mean grain count per labelled cell (± standard deviation)
Units	μ			
5	7.15	45.50	1	26.00
6	8.58	78.95	11	15.73 ± 5.87
7	10.01	125.00	56	19.09 ± 6.35
8	11.44	187.15	104	20.94 ± 7.47
9	12.87	265.85	63	23.05 ± 7.30
10	14.30	365.53	18	29.61 ± 10.74
11	15.73	485.59	4	29.25 ± 8.18
12	17.16	631.63	3	25.00 ± 6.25

APPENDIX TABLE 3

The effect of cell concentration on thymidine-H^3 uptake by mouse thymocytes *in vitro*: an incorporation period of 35 min was allowed for the thymidine-H^3

No. of cells per ml. nutrient medium ($\times 10^6$)	Grains per labelled cell (± standard deviation)	Percentage of cells labelled (± standard deviation)
21.6	15.63 ± 3.93	7.7 ± 2.9
12.8	24.07 ± 6.20	7.8 ± 1.4
3.2	41.00 ± 13.27	14.0 ± 3.2
2.0	38.23 ± 13.55	11.4 ± 2.0
1.4	32.5 ± 9.60	10.6 ± 2.9
0.8	56.20 ± 15.62	14.6 ± 2.5

APPENDIX TABLE 4

Rate of thymidine-H^3 uptake by mouse thymocytes *in vitro*: 4.4×10^6 cells / ml. nutrient medium

Incubation time (min.)	Grains per 100 cells
15	18.09
30	94.47
60	163.74
120	410.96
180	559.57

APPENDIX TABLE 5

The effect on labelling of a time lag at 37° C between the preparation of a thymocyte suspension and incubation with thymidine-H^3 (2 experiments): an incorporation period of 1 hr. was allowed for the thymidine-H^3

Time lag (hr.)	Grains per labelled cell (± standard deviation)	Percentage of cells labelled (± standard deviation)
0	60.7 ± 19.2	6.8 ± 1.8
½	64.1 ± 29.1	7.2 ± 1.6
1	60.3 ± 16.5	7.6 ± 2.3
1½	60.8 ± 14.2	7.2 ± 3.4
2	60.8 ± 19.6	5.0 ± 2.0
2½	56.6 ± 17.7	7.2 ± 2.9
3	71.0 ± 36.2	7.2 ± 4.7
0	69.0 ± 31.0	6.1 ± 2.6
1½	65.8 ± 16.6	6.6 ± 2.2
3	Specimen lost	6.1 ± 2.4
5½	37.9 ± 17.8	1.7 ± 1.2
25	Unlabelled	Unlabelled

APPENDIX TABLE 6

The uptake of tyrosine-H^3 by mouse thymocytes *in vitro*

Tyrosine-H^3 concentration (µc/ml. cell suspension)	Incubation time (min.)			
	30	55	79	103
	Percentage of cells labelled (± standard deviation)			
0.1	57.4 ± 6.1	85.4 ± 2.7	94.2 ± 3.0	97.0 ± 2.0
1	91.0 ± 3.7	97.0 ± 0.7	97.6 ± 1.5	98.6 ± 1.3

APPENDIX TABLE 7

The uptake of leucine-H^3 by mouse thymocytes *in vitro*

Leucine-H^3 concentration (µc/ml. cell suspension)	Incubation time (min.)			
	33	56	92	123
	Grains per labelled cell (± standard deviation)			
1	11.1 ± 5.7	14.2 ± 9.1	18.2 ± 12.5	14.8 ± 12.8
10	15.3 ± 15.3	38.8 ± 36.1	39.5 ± 32.3	42.5 ± 20.2
	Percentage of cells labelled (± standard deviation)			
1	56.6 ± 4.8	79.8 ± 4.6	94.6 ± 3.6	94.2 ± 1.5
10	97.4 ± 0.9	97.0 ± 1.6	99.2 ± 0.4	98.8 ± 1.8

APPENDIX TABLE 8

Correlation between thymocyte size and the grain number over cells labelled *in vitro* with leucine-H^3

Cell diameter		Radius^3	No. of cells	Mean grain count per labelled cell
Units	μ	(μ^3)	Total 238	(± standard deviation)
4.5	5.98	26.73	3	9.33 ± 2.52
5	6.65	36.59	64	10.87 ± 5.59
5.5	7.31	48.63	19	14.32 ± 6.35
6	7.98	63.52	70	18.86 ± 10.67
6.5	8.64	80.62	13	30.46 ± 9.29
7	9.31	100.54	45	32.18 ± 11.00
7.5	9.97	123.51	9	34.11 ± 10.22
8	10.64	150.57	15	39.13 ± 14.27

APPENDIX TABLE 9

Correlation between thymocyte size and the grain number over cells labelled *in vitro* with tyrosine-H^3

Cell diameter		Radius^3 (μ^3)	No. of cells Total 113	Mean grain count per labelled cell (± standard deviation)
Units	μ			
7	6.57	35.38	2	18.00 ± 15.56
7.5	7.03	43.58	4	7.00 ± 2.16
8	7.50	52.76	50	7.74 ± 3.05
8.5	7.97	63.46	10	10.40 ± 4.69
9	8.44	75.17	32	11.09 ± 7.53
10	9.38	103.16	7	28.71 ± 11.43
11	10.32	137.36	4	32.75 ± 10.53
12	11.26	178.39	3	38.67 ± 23.50
12.5	11.72	201.30	1	82.00

APPENDIX TABLE 10

The effect of nitrogen mustard (diluted in saline) on thymidine-H^3 uptake by mouse thymocytes *in vitro*: an incorporation period of 30 min. was allowed for the thymidine-H^3

Pretreatment time (hr.)	µg. nitrogen mustard / 4 x 10^6 cells				
	0	6.25	12.5	25	100
	Grains per labelled cell (± standard deviation)				
0	29.8 ± 13.4	33.4 ± 11.5	23.8 ± 10.2	30.0 ± 10.3	15.6 ± 5.8
½		36.2 ± 12.2	22.0 ± 11.0	17.8 ± 10.7	Unlabelled
1		21.8 ± 10.5	7.6 ± 3.8	Unlabelled	Unlabelled
1½	44.3 ± 17.6	6.3 ± 3.4	Unlabelled	Unlabelled	Unlabelled
	Percentage of cells labelled (± standard deviation)				
0	6.9 ± 3.1	9.4 ± 3.9	9.3 ± 3.1	10.2 ± 3.1	6.5 ± 2.4
½		11.3 ± 4.4	7.4 ± 2.7	5.4 ± 2.0	Unlabelled
1		6.6 ± 3.0	2.4 ± 1.6	Unlabelled	Unlabelled
1½	11.8 ± 3.8	2.7 ± 1.5	Unlabelled	Unlabelled	Unlabelled

APPENDIX TABLE 11

The effect of mannitol myleran on thymidine-H^3 uptake by mouse thymocytes *in vitro*: an incorporation period of 35 min. was allowed for the thymidine-H^3

Pretreatment time (hr.)	μg. mannitol myleran / 3 x 10^6 cells				
	0	50	500	1000	2000
	Grains per labelled cell (± standard deviation)				
0	50.5 ± 16.1	52.8 ± 16.7	40.0 ± 20.6	46.8 ± 12.9	46.6 ± 13.4
¾	38.0 ± 13.9	41.5 ± 14.8	45.0 ± 17.4	41.8 ± 16.5	30.1 ± 10.9
1½	34.1 ± 13.8	29.1 ± 12.1	32.2 ± 16.9	30.8 ± 14.6	26.6 ± 14.9
	Percentage of cells labelled (± standard deviation)				
0	7.7 ± 2.3	10.2 ± 3.0	10.6 ± 2.5	8.3 ± 1.7	10.1 ± 2.3
¾	10.6 ± 2.0	10.8 ± 2.6	9.8 ± 2.1	9.1 ± 2.0	9.3 ± 2.1
1½	9.4 ± 2.4	7.0 ± 1.9	9.8 ± 2.0	9.4 ± 2.1	8.5 ± 2.0

APPENDIX TABLE 12

The effect of chlorambucil on thymidine-H^3 uptake by mouse thymocytes *in vitro*: an incorporation period of 35 min. was allowed for the thymidine-H^3

Pretreatment time (hr.)	µg. chlorambucil / 3 x 10^6 cells				
	0	100	300	400	500
	Grains per labelled cell (± standard deviation)				
0	16.3 ± 8.0	17.6 ± 8.5	9.8 ± 4.4	7.6 ± 2.6	Unlabelled
¾	20.8 ± 9.5	15.6 ± 9.0	8.7 ± 3.6	6.9 ± 2.3	Unlabelled
1½	12.4 ± 5.9	17.0 ± 10.7	9.9 ± 3.8	7.1 ± 2.0	Unlabelled
	Percentage of cells labelled (± standard deviation)				
0	10.2 ± 1.6	12.0 ± 1.3	8.3 ± 1.5	6.3 ± 1.5	Unlabelled
¾	10.8 ± 1.8	10.7 ± 2.3	9.5 ± 0.9	8.4 ± 0.3	Unlabelled
1½	11.3 ± 1.3	9.5 ± 0.6	11.1 ± 1.6	5.1 ± 1.0	Unlabelled

APPENDIX TABLE 13

The effect of tretamine on thymidine-H^3 uptake by mouse thymocytes *in vitro*: an incorporation period of 35 min. was allowed for the thymidine-H^3

Pretreatment time (hr.)	µg. tretamine / 3 x 10^6 cells				
	0	50	250	500	1000
	Grains per labelled cell (± standard deviation)				
0	57.9 ± 18.9	54.5 ± 19.2	44.2 ± 13.7	33.3 ± 15.2	28.3 ± 9.5
¾	45.2 ± 13.7	48.4 ± 16.4	43.1 ± 16.4	32.8 ± 13.2	13.3 ± 5.1
1½	30.8 ± 11.7	43.4 ± 18.9	18.9 ± 9.3	16.3 ± 6.8	Unlabelled
	Percentage of cells labelled (± standard deviation)				
0	7.1 ± 1.6	8.9 ± 1.8	8.5 ± 2.1	5.3 ± 2.0	9.4 ± 2.1
¾	10.3 ± 2.6	9.5 ± 2.1	11.2 ± 2.7	9.7 ± 1.7	7.6 ± 2.1
1½	9.2 ± 2.2	6.4 ± 1.7	5.7 ± 2.0	5.9 ± 2.1	Unlabelled

APPENDIX TABLE 14

The effect of nitrogen mustard (diluted in water) on thymidine-H^3 uptake by mouse thymocytes in vitro: an incorporation period of 35 min. was allowed for the thymidine-H^3

Pretreatment time (hr.)	µg. nitrogen mustard / 3 x 10^6 cells				
	0	5	10	100	500
	Grains per labelled cell (± standard deviation)				
0	56.0 ± 16.4	46.6 ± 12.1	39.3 ± 13.7	16.7 ± 5.5	11.9 ± 5.3
¾	64.4 ± 19.9	11.5 ± 6.7	Unlabelled	Unlabelled	Unlabelled
1½	50.7 ± 22.6	Unlabelled	Unlabelled	Unlabelled	Unlabelled
	Percentage of cells labelled (± standard deviation)				
0	16.3 ± 3.4	14.8 ± 2.4	12.0 ± 3.0	13.9 ± 3.2	10.9 ± 2.7
¾	17.7 ± 4.7	6.3 ± 1.8	Unlabelled	Unlabelled	Unlabelled
1½	14.8 ± 2.3	Unlabelled	Unlabelled	Unlabelled	Unlabelled

APPENDIX TABLE 15

The effect of thioTEPA on thymidine-H^3 uptake by mouse thymocytes in vitro: an incorporation period of 35 min. was allowed for the thymidine-H^3

Pretreatment time (hr.)	µg. thioTEPA / 3 x 10^6 cells			
	0	500	1000	2000
	Grains per labelled cell (± standard deviation)			
0	45.2 ± 15.6	46.2 ± 13.4	37.1 ± 14.1	Unlabelled
¾	56.0 ± 23.4	57.0 ± 20.5	16.2 ± 11.6	Unlabelled
1½	43.7 ± 15.4	45.8± 14.4	Unlabelled	Unlabelled
	Percentage of cells labelled (± standard deviation)			
0	13.1 ± 2.3	16.0 ± 2.7	13.0 ± 3.0	Unlabelled
¾	13.1 ± 2.1	8.9 ± 3.1	7.3 ± 1.8	Unlabelled
1½	12.5 ± 2.3	15.0 ± 3.4	Unlabelled	Unlabelled

APPENDIX TABLE 16

The effect of melphalan on thymidine-H^3 uptake by mouse thymocytes in vitro: an incorporation period of 35 min. was allowed for the thymidine-H^3

Pretreatment time (hr.)	µg. melphalan / 3 x 10^6 cells				
	0	10	100	500	1300
	Grains per labelled cell (± standard deviation)				
0	21.1 ± 6.9	23.6 ± 9.5	18.9 ± 7.1	15.2 ± 6.5	15.8 ± 5.9
¾	20.3 ± 10.6	22.1 ± 9.4	23.3 ± 10.3	13.2 ± 6.3	Unlabelled
1½	29.0 ± 17.5	24.1 ± 12.4	26.2 ± 15.8	Unlabelled	Unlabelled
	Percentage of cells labelled (± standard deviation)				
0	5.4 ± 1.4	5.3 ± 1.1	5.5 ± 1.5	5.9 ± 1.2	5.8 ± 1.0
¾	7.6 ± 1.2	6.4 ± 0.8	7.3 ± 0.4	6.7 ± 1.6	Unlabelled
1½	3.8 ± 0.8	5.7 ± 0.7	5.4 ± 1.1	Unlabelled	Unlabelled

APPENDIX TABLE 17

The effect of nitrogen mustard on uridine-H^3 uptake by mouse thymocytes in vitro: an incorporation period of 35 min. was allowed for the uridine-H^3

Pretreatment time (hr.)	µg. nitrogen mustard / 11 x 10^6 cells				
	0	5	10	100	500
	Grains per labelled cell (± standard deviation)				
0	13.4 ± 7.0	10.0 ± 4.9	12.4 ± 6.4	10.3 ± 5.6	7.0 ± 2.2
1½	14.4 ± 6.5	Negligible labelling	Unlabelled	Unlabelled	Unlabelled
	Percentage of cells labelled (± standard deviation)				
0	62.6 ± 4.9	41.7 ± 6.3	43.2 ± 12.9	52.1 ± 4.6	31.7 ± 4.1
1½	82.4 ± 4.5	3.5 ± 2.3	Unlabelled	Unlabelled	Unlabelled

APPENDIX TABLE 18

The effect of melphalan on uridine-H^3 uptake by mouse thymocytes *in vitro*: an incorporation period of 35 min. was allowed for the uridine-H^3

Pretreatment time (hr.)	µg. melphalan / 3 x 10^6 cells				
	0	5	10	100	1000
	Grains per labelled cell (± standard deviation)				
0	12.2 ± 6.0	15.1 ± 8.2	9.7 ± 4.7	10.7 ± 4.1	9.4 ± 3.6
¾	13.1 ± 8.3	13.2 ± 9.0	8.3 ± 3.5	Unlabelled	Unlabelled
1½	8.9 ± 4.6	8.8 ± 4.0	Unlabelled	Unlabelled	Unlabelled
	Percentage of cells labelled (± standard deviation)				
0	66.0 ± 3.9	66.8 ± 2.9	68.8 ± 8.9	62.8 ± 6.8	37.0 ± 6.0
¾	26.8 ± 4.7	12.8 ± 3.0	12.0 ± 4.0	Unlabelled	Unlabelled
1½	7.8 ± 3.2	4.4 ± 2.0	Unlabelled	Unlabelled	Unlabelled

APPENDIX TABLE 19

The effect of chlorambucil on uridine-H^3 uptake by mouse thymocytes *in vitro*: an incorporation period of 35 min. was allowed for the uridine-H^3

Pretreatment time (hr.)	µg. chlorambucil / 9 x 10^6 cells				
	0	100	300	400	500
	Grains per labelled cell (± standard deviation)				
0	11.9 ± 7.1	Not done	6.0 ± 1.8	5.4 ± 1.5	5.8 ± 1.8
1½	8.3 ± 3.7	9.3 ± 4.5	8.1 ± 3.4	7.6 ± 3.9	7.4 ± 4.6
	Percentage of cells labelled (± standard deviation)				
0	69.2 ± 4.8	Not done	8.3 ± 2.9	2.2 ± 1.7	2.6 ± 1.3
1½	16.7 ± 5.0	23.7 ± 5.7	14.2 ± 2.7	11.8 ± 3.1	7.8 ± 3.1

APPENDIX TABLE 20

The effect of mannitol myleran on uridine-H^3 uptake by mouse thymocytes *in vitro*: an incorporation period of 35 min. was allowed for the uridine-H^3

Pretreatment time (hr.)	µg. mannitol myleran / 3 x 10^6 cells				
	0	50	500	1000	2000
	Grains per labelled cell (± standard deviation)				
0	13.6 ± 13.1	17.1 ± 16.8	16.6 ± 10.4	17.4 ± 15.3	15.1 ± 18.5
¾	16.1 ± 14.8	14.0 ± 9.7	21.5 ± 23.4	14.5 ± 13.2	16.3 ± 17.9
1½	15.8 ± 10.7	15.9 ± 10.8	13.8 ± 11.5	15.8 ± 11.4	Specimen lost
	Percentage of cells labelled (± standard deviation)				
0	53.6 ± 5.0	57.6 ± 4.6	72.0 ± 10.2	60.8 ± 2.3	55.0 ± 4.1
¾	69.0 ± 4.4	38.2 ± 11.9	56.4 ± 5.5	72.8 ± 7.8	75.6 ± 3.6
1½	62.2 ± 9.0	51.0 ± 4.4	25.8 ± 11.3	64.4 ± 5.7	38.8 ± 3.4

APPENDIX TABLE 21

The effect of methotrexate on thymidine-H^3 uptake by mouse thymocytes *in vitro*: an incorporation period of 35 min. was allowed for the thymidine-H^3

Pretreatment time (hr.)	µg. methotrexate / 3 x 10^6 cells					
	0	1	5	10	50	100
	Grains per labelled cell (± standard deviation)					
0	24.4 ± 12.0	23.6 ± 11.0	30.7 ± 10.2	38.9 ± 12.4	34.0 ± 11.4	35.5 ± 11.3
1½	27.6 ± 11.5	41.2 ± 13.6	40.8 ± 13.1	38.8 ± 11.8	50.3 ± 19.1	54.3 ± 16.7
	Percentage of cells labelled (± standard deviation)					
0	8.6 ± 2.7	8.6 ± 2.6	9.9 ± 2.8	8.6 ± 3.1	8.2 ± 3.2	8.6 ± 3.6
1½	10.4 ± 2.8	11.0 ± 3.0	11.8 ± 4.2	9.2 ± 3.1	10.7 ± 2.5	9.7 ± 3.4

APPENDIX TABLE 22

The effect of methotrexate on uridine-H^3 uptake by mouse thymocytes in vitro: An incorporation period of 35 min. was allowed for the uridine-H^3

Pretreatment time (hr.)	µg. methotrexate / 3 x 10^6 cells					
	0	1	5	10	50	100
	Grains per labelled cell (± standard deviation)					
0	13.3± 8.5	10.6± 5.6	9.4± 4.7	11.5± 7.2	10.3± 5.7	10.5± 4.6
1½	11.3± 7.6	11.1± 6.8	12.8± 8.3	14.4± 12.2	18.4± 11.8	12.5± 11.5

APPENDIX TABLE 23

The effect of nitrogen mustard on tyrosine-H^3 uptake by mouse thymocytes *in vitro*: an incorporation period of 35 min. was allowed for the tyrosine-H^3

Pretreatment time (hr.)	µg. nitrogen mustard / 3 x 10^6 cells				
	0	5	10	100	500
	Grains per labelled cell (± standard deviation)				
0	15.9 ± 13.1	12.4 ± 9.5	13.3 ± 10.4	10.5 ± 4.0	7.2 ± 2.2
¾	16.3 ± 12.0	12.2 ± 6.8	16.0 ± 8.3	Unlabelled	Unlabelled
1½	18.8 ± 13.2	8.8 ± 3.7	Unlabelled	Unlabelled	Unlabelled
	Percentage of cells labelled (± standard deviation)				
0	87.7 ± 3.2	81.1 ± 3.3	71.3 ± 6.7	36.5 ± 6.3	23.3 ± 4.7
¾	89.1 ± 2.1	43.0 ± 2.3	35.2 ± 5.4	Unlabelled	Unlabelled
1½	92.4 ± 3.2	24.9 ± 3.2	3.7 ± 1.6	Unlabelled	Unlabelled

APPENDIX TABLE 24

The effect of thioTEPA on tyrosine-H^3 uptake by mouse thymocytes *in vitro*: an incorporation period of 35 min. was allowed for the tyrosine-H^3

Pretreatment time (hr.)	μg. thioTEPA / 3 x 10^6 cells				
	0	500	1000	2000	3000
	Grains per labelled cell (± standard deviation)				
0	11.4 ± 5.6	11.3 ± 4.7	6.7 ± 2.8	Unlabelled	Unlabelled
1½	14.0 ± 9.5	9.2 ± 6.2	Unlabelled	Unlabelled	Unlabelled
	Percentage of cells labelled (± standard deviation)				
0	74.8 ± 5.4	63.2 ± 5.2	16.2 ± 6.6	2.2 ± 1.9	Unlabelled
1½	82.6 ± 5.6	46.6 ± 3.2	Unlabelled	Unlabelled	Unlabelled

APPENDIX TABLE 25

The effect of tretamine on tyrosine-H^3 uptake by mouse thymocytes *in vitro*: an incorporation period of 35 min. was allowed for the tyrosine-H^3

Pretreatment time (hr.)	µg. tretamine / 3 x 10^6 cells				
	0	50	250	500	1000
	Grains per labelled cell (± standard deviation)				
0	13.6 ± 9.6	15.2 ± 9.0	15.7 ± 9.9	10.7 ± 6.8	14.7 ± 9.7
¾	22.9 ± 11.3	17.0 ± 9.1	27.6 ± 16.4	17.7 ± 10.5	21.8 ± 13.2
1½	24.2 ± 17.2	17.0 ± 15.8	21.7 ± 10.2	18.8 ± 8.1	13.4 ± 6.6
	Percentage of cells labelled (± standard deviation)				
0	95.2 ± 1.8	87.2 ± 4.4	95.8 ± 1.3	91.2 ± 4.0	90.6 ± 2.6
¾	96.2 ± 2.6	96.4 ± 0.5	95.2 ± 1.8	92.0 ± 2.1	87.4 ± 3.4
1½	94.8 ± 1.6	92.8 ± 2.4	82.8 ± 2.5	64.8 ± 4.2	30.2 ± 4.9

APPENDIX TABLE 26

The effect of mannitol myleran on tyrosine-H^3 uptake by mouse thymocytes in vitro: an incorporation period of 35 min. was allowed for the tyrosine-H^3

Pretreatment time (hr.)	µg. mannitol myleran / 3 x 10^6 cells				
	0	50	500	1000	2000
	Grains per labelled cell (± standard deviation)				
0	10.2 ± 4.8	14.4 ± 7.6	9.0 ± 3.2	10.9 ± 6.5	12.4 ± 6.7
¾	14.2 ± 7.3	12.5 ± 5.9	12.2 ± 5.5	11.5 ± 6.3	10.9 ± 4.4
1½	12.9 ± 7.1	12.4 ± 7.0	13.7 ± 7.5	14.5 ± 9.0	10.8 ± 4.1
	Percentage of cells labelled (± standard deviation)				
0	63.2 ± 3.7	74.2 ± 5.8	71.4 ± 2.2	73.0 ± 2.0	83.8 ± 4.3
¾	89.0 ± 1.9	55.4 ± 9.2	88.8 ± 5.1	88.0 ± 2.0	90.4 ± 1.5
1½	91.0 ± 1.6	83.6 ± 6.1	87.2 ± 2.7	84.2 ± 3.8	91.0 ± 2.3

APPENDIX TABLE 27

The effect of melphalan on the uptake of tyrosine-H^3 (5 μc/ml.) by mouse thymocytes *in vitro*: an incorporation period of 35 min. was allowed for the tyrosine-H^3

Pretreatment time (hr.)	μg. melphalan / 3 x 10^6 cells				
	0	5	10	100	1000
	Grains per labelled cell (± standard deviation)				
0	14.4 ± 9.9	14.8 ± 9.0	14.2 ± 7.0	12.9 ± 6.9	10.1 ± 4.5
1½	28.3 ± 14.4	33.0 ± 23.1	32.3 ± 17.0	12.9 ± 3.8	Unlabelled
	Percentage of cells labelled (± standard deviation)				
0	25.9 ± 6.7	42.8 ± 12.0	22.9 ± 6.9	49.7 ± 5.6	21.3 ± 5.2
1½	23.6 ± 6.1	23.9 ± 5.9	26.7 ± 5.2	6.2 ± 3.8	Unlabelled

APPENDIX TABLE 28

The effect of phenylalanine on the uptake of tyrosine-H^3 (1 µc/ml.) by mouse thymocytes *in vitro*: an incorporation period of 35 min. was allowed for the tyrosine-H^3

Pretreatment time (hr.)	µg. phenylalanine / 3 x 10^6 cells				
	0	2.7	5.4	54	541
	Grains per labelled cell (± standard deviation)				
0	12.6 ± 5.6	11.0 ± 3.6	12.5 ± 4.9	10.9 ± 7.4	10.3 ± 2.6
1½	13.6 ± 6.6	13.6 ± 5.6	15.7 ± 7.8	13.3 ± 5.7	10.5 ± 4.1
	Percentage of cells labelled (± standard deviation)				
0	44.4 ± 8.6	47.6 ± 4.6	57.8 ± 1.8	37.4 ± 5.9	18.4 ± 7.2
1½	67.2 ± 5.9	56.2 ± 3.4	57.0 ± 6.9	71.8 ± 7.8	33.6 ± 4.0

APPENDIX TABLE 29

The effect of phenylalanine on leucine-H^3 uptake by mouse thymocytes in vitro: an incorporation period of 35 min. was allowed for the leucine-H^3

Pretreatment time (hr.)	µg. phenylalanine / 3 x 10^6 cells				
	0	2.7	5.4	54	541
	Grains per labelled cell (± standard deviation)				
1½	18.3 ± 10.2	17.4 ± 8.6	17.7 ± 10.7	16.0 ± 9.0	16.9 ± 9.3
	Percentage of cells labelled (± standard deviation)				
1½	88.0 ± 4.7	96.6 ± 1.1	94.0 ± 2.2	97.4 ± 1.1	92.6 ± 3.3

APPENDIX TABLE 30

The effect of melphalan on leucine-H^3 uptake by mouse thymocytes *in vitro*: an incorporation period of 43 min. was allowed for the leucine-H^3

Pretreatment time (hr.)	µg. melphalan / 3 x 10^6 cells				
	0	5	10	100	1000
	Percentage of cells labelled (± standard deviation)				
0	21.0 ± 3.8	13.8 ± 5.4	31.2 ± 6.0	2.4 ± 1.5	0.6 ± 1.3
¾	20.0 ± 6.1	1.8 ± 1.3	12.2 ± 8.0	8.7 ± 3.1	Unlabelled
1½	8.6 ± 1.8	0.8 ± 0.8	7.2 ± 1.6	Unlabelled	Unlabelled

APPENDIX TABLE 31

The effect of nitrogen mustard on leucine-H^3 uptake by mouse thymocytes in vitro: an incorporation period of 30 min. was allowed for the leucine-H^3

Pretreatment time (hr.)	µg. nitrogen mustard / 3 x 10^6 cells				
	0	5	10	100	500
	Percentage of cells labelled (± standard deviation)				
0	93.8 ± 3.1	89.6 ± 1.8	88.0 ± 4.4	77.6 ± 2.3	42.2 ± 4.8
¾	94.8 ± 2.6	63.2 ± 5.6	36.2 ± 6.1	2.2 ± 1.8	Unlabelled
1½	72.0 ± 6.7	18.6 ± 2.7	Unlabelled	Unlabelled	Unlabelled

APPENDIX TABLE 32

The effect of nitrogen mustard on tyrosine-H^3 uptake by mouse thymocytes of different sizes

Cell diameter		Number of cells	Mean grain count per labelled cell	Standard deviation
Units	μ			
	Diluent control, without pretreatment Total 316 cells			
4	5.7	5	7.8	2.4
4.5	6.4	11	6.3	2.0
5	7.1	110	9.0	3.7
5.5	7.8	48	11.2	4.2
6	8.5	87	15.2	8.4
7	10.0	28	25.9	10.2
7.5	10.7	8	28.9	8,5
8	11.4	6	24.2	10.6
8.5	12.1	1	12	-
	10μg. nitrogen mustard, ¾ hr. pretreatment Total 225 cells			
5	6.0	2	5.5	0.7
6	7.1	78	6.7	3.3
7	8.3	74	11.4	5.5
8	9.5	58	15.6	6.0
9	10.7	11	18.4	6.4
10	11.9	2	31.0	1.4

Continued..

Appendix Table 32 continued

Cell diameter		Number of cells	Mean grain count per labelled cell	Standard deviation
Units	μ			
500 μg. nitrogen mustard, without pretreatment Total 307 cells				
4.5	6.4	1	7	-
5	7.1	18	6.6	2.4
5.5	7.8	3	4	0
6	8.5	48	5.9	2.3
6.5	9.3	13	8.1	3.2
7	10.0	29	7.3	3.0
7.5	10.7	3	9.7	2.1
8	11.4	35	6.5	3.6
8.5	12.1	4	8.2	2.9
9	12.8	40	5.9	2.2
9.5	13.5	2	5.5	0.7
10	14.2	75	8.5	4.0
11	15.7	21	10.4	5.2
12	17.1	11	9.8	4.1
12.5	17.8	1	8	-
13	18.5	3	10.7	8.1

APPENDIX TABLE 33

The effect of nitrogen mustard on leucine-H^3 uptake by mouse thymocytes of different sizes

Cell diameter		Number of cells	Mean grain count per labelled cell	Standard deviation
Units	μ			
	Diluent control, without pretreatment Total 259 cells			
4	5.8	1	4	-
5	7.2	3	6.3	1.5
5.5	7.9	9	9.0	3.7
6	8.6	121	9.7	4.1
6.5	9.4	84	13.4	5.9
7	10.1	36	18.2	8.6
7.5	10.8	3	23.7	11.1
8	11.5	2	40.0	8.5
	10 μg. nitrogen mustard, ¾ hr. pretreatment Total 259 cells			
5	7.2	5	6.6	3.1
5.5	7.9	26	6.4	1.9
6	8.6	155	7.3	3.2
6.5	9.4	36	12.7	10.9
7	10.1	23	14.8	9.0
7.5	10.8	5	14.6	6.9
8	11.5	6	18.0	8.6
8.5	12.2	1	7.0	-
10	14.4	2	10.0	4.2

Continued..

Appendix Table 33 continued

Cell diameter		Number of cells	Mean grain count per labelled cell	Standard deviation
Units	μ			
500 μg. nitrogen mustard, without pretreatment Total 259 cells				
5.5	7.9	1	4.0	-
6	8.6	86	6.5	1.8
6.5	9.4	67	6.6	2.0
7	10.1	66	6.7	2.4
7.5	10.8	12	8.8	3.1
8	11.5	20	12.2	8.7
8.5	12.2	2	17.0	0
9	12.9	1	11.0	-
10	14.4	2	14.5	10.6
12	17.3	1	13.0	-
15	21.6	1	17.0	-

APPENDIX TABLE 34

The effect of nitrogen mustard (500 µg./ml.) on the concentration of mouse thymocytes in vitro

Incubation time (min.)	Cell number / ml. ($\times 10^4$)	
	Control	500 µg. nitrogen mustard / ml.
0	244	244
8	209	
14		227
21	210	
27		207
33	218	
40		217
45	225	
65		187
71	162	
77		150
83	114	

APPENDIX TABLE 35

The effect of nitrogen mustard on labelling of mouse thymocytes previously incubated with thymidine-H^3

Incubation time after addition of nitrogen mustard (hr.)	μg. nitrogen mustard / 3 x 10^6 cells		
	0	10	100
	Grains per labelled cell (± standard deviation)		
½	57.0 ± 18.2	59.9 ± 21.6	34.0 ± 12.0
1	69.2 ± 17.9	53.5 ± 22.2	33.1 ± 14.0
1½	75.5 ± 23.9	54.8 ± 21.3	37.4 ± 19.0

APPENDIX TABLE 36

The effect of nitrogen mustard on the cell size of thymocytes previously labelled with thymidine-H^3 and of unlabelled thymocytes: the diameters were measured of 182 cells selected at random

Cell diameter		Number of cells			
		Control		100 µg. nitrogen mustard	
Units	µ	Labelled	Unlabelled	Labelled	Unlabelled
4	5.6	0	2	0	0
5	7.2	0	116	0	91
6	8.6	2	27	4	58
7	10.0	23	1	14	0
8	11.4	6	3	7	2
9	12.9	1	1	2	1
10	14.3	0	0	2	0
11	15.7	0	0	1	0
Total		32	150	30	152

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Effects of Alkylating Agents on Thymidine Uptake by Mouse Thymocytes with Reference to an *in vitro* Sensitivity Test for Individual Human Tumours

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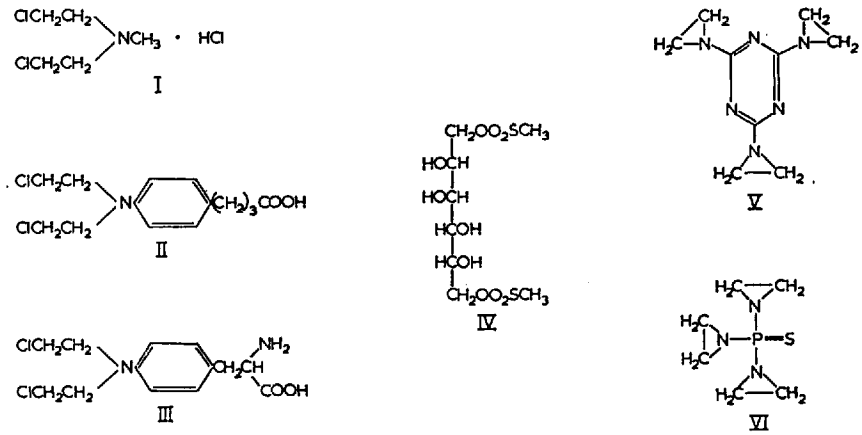
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INTRODUCTION

IN RECENT years, there have been many attempts to find an *in vitro* method of determining the *in vivo* sensitivity of a given tumour in a given patient to various chemotherapeutic agents. Most of the proposed methods involve the incubation of small pieces of the tumour (or effusions) with various drugs, followed by an estimation of cell damage. The assumption is made that the drug most effective in damaging the tumour piece *in vitro* would be the most effective against the tumour in the patient, due allowance first being made for differences in drug toxicities. Damage may be assessed from changes in cell counts during culture [1], from cytological changes [2], or from biochemical analyses on the whole cell population with determination of protein content [3], dehydrogenase activity [4], or oxygen consumption [5]. Drug action on individual cells can be estimated from the two former methods but tissue culture over several days is usually involved and selection of the most resistant cell type may occur. Major disadvantages of the biochemical methods are that normal or necrotic areas within the tumour piece influence results and variations from cell to cell cannot be measured.

Wolberg and Curreri [6] and Wolberg and Brown [7] studied the effect of cancer chemotherapeutic agents on nucleic acid synthesis in slices of biopsied tumours. By following the uptake of tritiated thymidine or uridine autoradiographically, they obtained information on growth inhibition at the cellular level. Each tumour slice was incubated in tissue culture medium at 37°C with one of the drugs for $\frac{1}{2}$ or 1 hr. One of the labelled nucleosides was then added and incubation continued for a further $17\frac{1}{2}$ or 23 hr. The tissue was fixed, sectioned and exposed to autoradiographic stripping film. The effect of each drug on labelling of DNA and RNA was determined from grain counts. Similar work [8] was carried out on Ehrlich ascites cells exposed to drugs for 24 hr and to tritiated thymidine for a further hour. The advantage of examining uptake of labelled metabolites autoradiographically is that the response of individual cells, both neoplastic and normal, can be determined. These workers did not investigate in detail the effect of varying the time of pre-incubation with the drug. The following work shows that the apparent effectiveness of a drug depends on the time it acts on the cells and that this time-effect varies from one drug to another.

The importance of the incubation times in such an *in vitro* test was investigated on a standard cell system. Mouse thymus was chosen as this standard tissue because it consists of few cell types and contains cells with a high mitotic index.



Structures I-VI

MATERIALS AND METHOD

Mouse thymocytes

Female A2G mice, approximately 30 days old and weighing 9·3 – 15·6 g were obtained from Glaxo Laboratories, Harefield. A separate mouse was used for each drug assay. The mouse was killed with anaesthetic ether and its thymus aseptically removed into approximately 15 ml tissue culture medium. The thymus was teased into a cell suspension which was filtered through gauze to remove cell clumps. The cell concentration was adjusted to 3×10^6 cells / ml.

Tissue culture medium

This was Hanks' balanced salt solution enriched with 12·5% pooled A2G mouse serum which had been previously heated at 56°C for 30 min. Penicillin and streptomycin were added to give a final concentration of approximately 100 and 75 Units respectively per ml.

Drugs

Nitrogen mustard (I), di-2-chloroethylmethylamine hydrochloride, "Mustine Hydrochloride" (Boots). Molecular weight 192·5. Batch number 324W.

Chlorambucil (II), *p*-(di-2-chloroethylamino) phenylbutyric acid, kindly supplied by Dr. D. A. G. Galton, Chester Beatty Research Institute, London. Molecular weight 304·2. Batch number 1389.

Melphalan (III), L-*p*-(di-2-chloroethylamino)phenylalanine, "Alkeran" (Burroughs Wellcome). Molecular weight 305·2. Batch number 50993.

Mannitol myleran (IV), 1,6-(dimethanesulphonyl)-1,6-dideoxymannitol, kindly supplied by Dr. G. M. Timmis, Chester Beatty Research Institute, London. Molecular weight 338.

Tretamine (V), triethylenemelamine, (ICI). Molecular weight 204·2. Batch number UP272.

ThioTEPA (VI), triethylenethiophosphortriamide, (Lederle). Molecular weight 189·22. Batch number 4650-137A.

Drug diluents

Pyrogen-free distilled water was used for nitrogen mustard, thioTEPA, tretamine and mannitol myleran. Chlorambucil and melphalan, being insoluble in water, were dissolved according to the manufacturer's instructions in ethyl alcohol and acid-alcohol respectively and diluted with propylene glycol diluent as supplied by the manufacturer. All the tubes in the chlorambucil and melphalan series were adjusted to contain the same alcohol (or acid-alcohol) and propylene glycol concentrations. To neutralize the acid, 0·05 ml 5% sodium bicarbonate was added to all tubes in the melphalan series.

Tritiated thymidine

Tritiated thymidine-6-T(n), batch 30, with a specific activity of 8·8c / mM was obtained from the Radiochemical Centre, Amersham. At the appropriate time, 0·1ml of thymidine solution containing 1 μc / ml was added to each tube to give a final concentration of 0·1 μc / 3×10^6 cells.

Assay procedure

For each assay, 1-ml aliquots of cell suspension were placed in fifteen 16 × 150 mm pyrex test tubes, which were divided into three groups of five. Four dilutions of each drug were made immediately before use and 0·1 ml

of each solution added to the appropriate tubes. One-tenth of a millilitre of drug diluent was added to the three remaining tubes as controls. Thymidine was added to the first group of tubes immediately after the drug, to the second group after the cells had been incubated at 37°C with the drug for 45 min and to the third group after the cells had been incubated with the drug for 1½ hr. Tubes were incubated for 35 min after the thymidine had been added.

The cells were then centrifuged at 4°C and 650 g for 7½ min. The cell deposit was washed twice in 5% bovine plasma albumin (Armour) in saline, and the final deposit in each tube was smeared on fat-free slides previously treated with a solution of 0·5% gelatin and 0·1% chrome alum. All smears were fixed in methanol overnight. It was assumed that all detected radioactivity was confined to freshly synthesized DNA, since fixation in absolute methanol for as short a time as 3 min is reported to remove all precursor pool tritium [9].

Autoradiographic procedure

Kodak A.R. 10 stripping film was left standing over a saturated calcium nitrate solution at 4°C before it was applied to the slides [10]. The autoradiographs were exposed at 4°C for 1 or 2 weeks before being processed with D19B developer and Fix-sol (Johnson). These exposure times were chosen for ease of counting. It was found that the percentage cells labelled was most easily counted on smears exposed for 2 weeks but, since this exposure time resulted in some labelled cells showing grains too numerous to be counted with accuracy, the mean number of grains per labelled cell was determined from smears exposed for the shorter time. These two batches of autoradiographs were given random code numbers before independent examination by two observers. Sixty labelled cells were examined on each autoradiograph exposed for 1 week to give a mean number of grains per labelled cell, and a total of 3000 cells was examined on each autoradiograph exposed for 2 weeks to give a mean value for the percentage cells labelled.

RESULTS

Grains per labelled cell (*Figs.* 1–6)

Five of the six alkylating agents examined reduced the mean number of grains over labelled cells. These agents were nitrogen mustard, tretamine, melphalan, thioTEPA and chlorambucil. There were wide differences in the drug concentrations needed to decrease the average grain count below the control level. For example, with 1½ hr pretreatment, 5μg nitrogen mustard (0·02mM) had the same effect on thymidine incorporation as 1 mg thioTEPA (4·40mM). Up to 4·9mM mannitol myleran had no effect on the grain count.

Percentage cells labelled (*Figs.* 7–12)

With the exception of mannitol myleran, all alkylating agents examined reduced the number of cells synthesizing DNA. Up to 4·9mM mannitol myleran had no effect on the number of labelled cells. In general, response curves showing the effect of the drugs on the percentage cells labelled were similar to the corresponding curves showing the drug effect on the mean number of grains over labelled cells. The curves for nitrogen mustard are an exception to this, in that, at low drug concentrations and with no pretreatment, the number of grains per labelled cell was reduced to a greater extent than the percentage cells labelled.

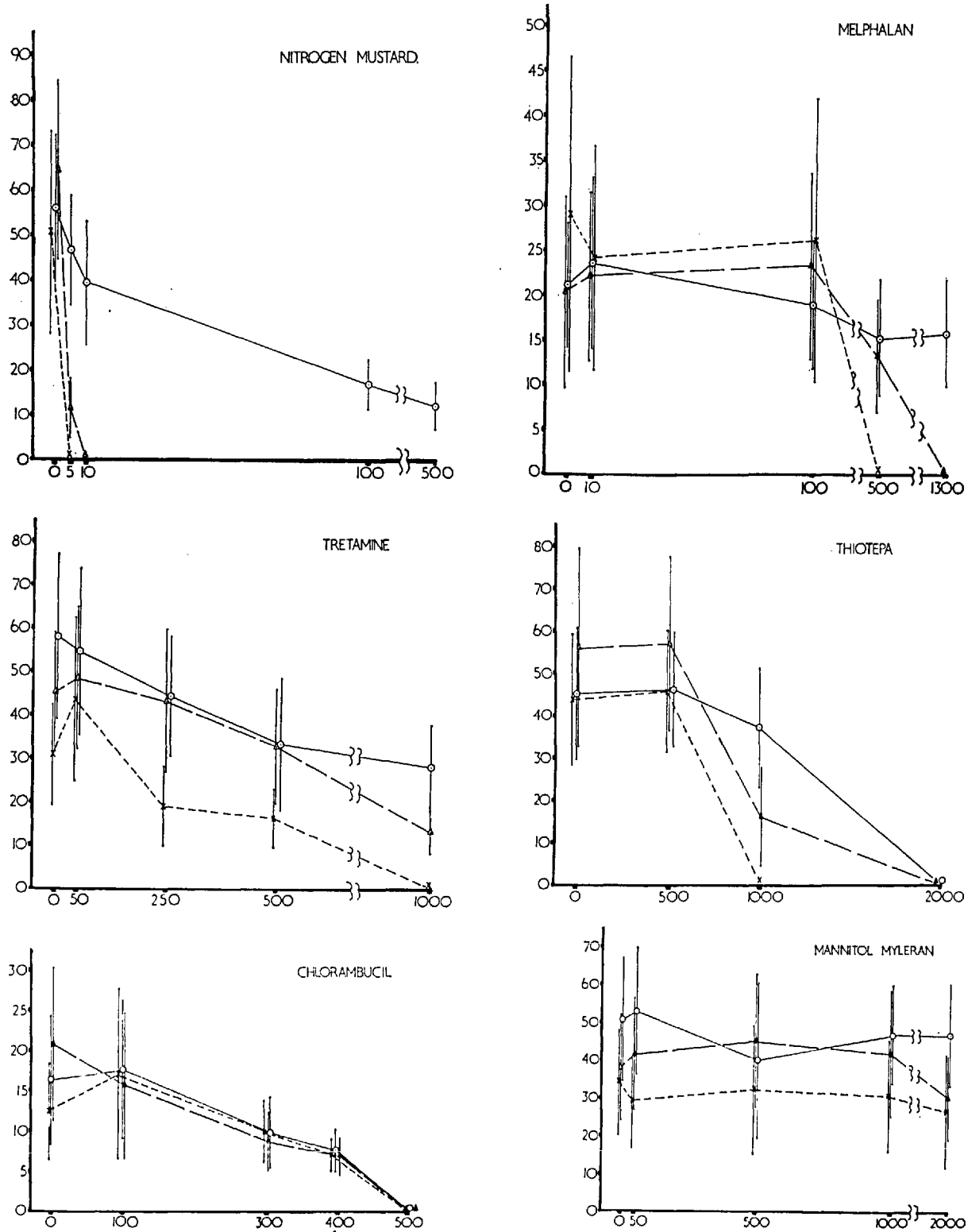
Chlorambucil and melphalan

In the chlorambucil and melphalan experiments, low grain counts per labelled cell and low percentages of labelled cells were found for cells incubated with the lower drug concentrations, and also for the diluent controls. This low level of labelling may have been due to the presence of propylene glycol.

Pretreatment

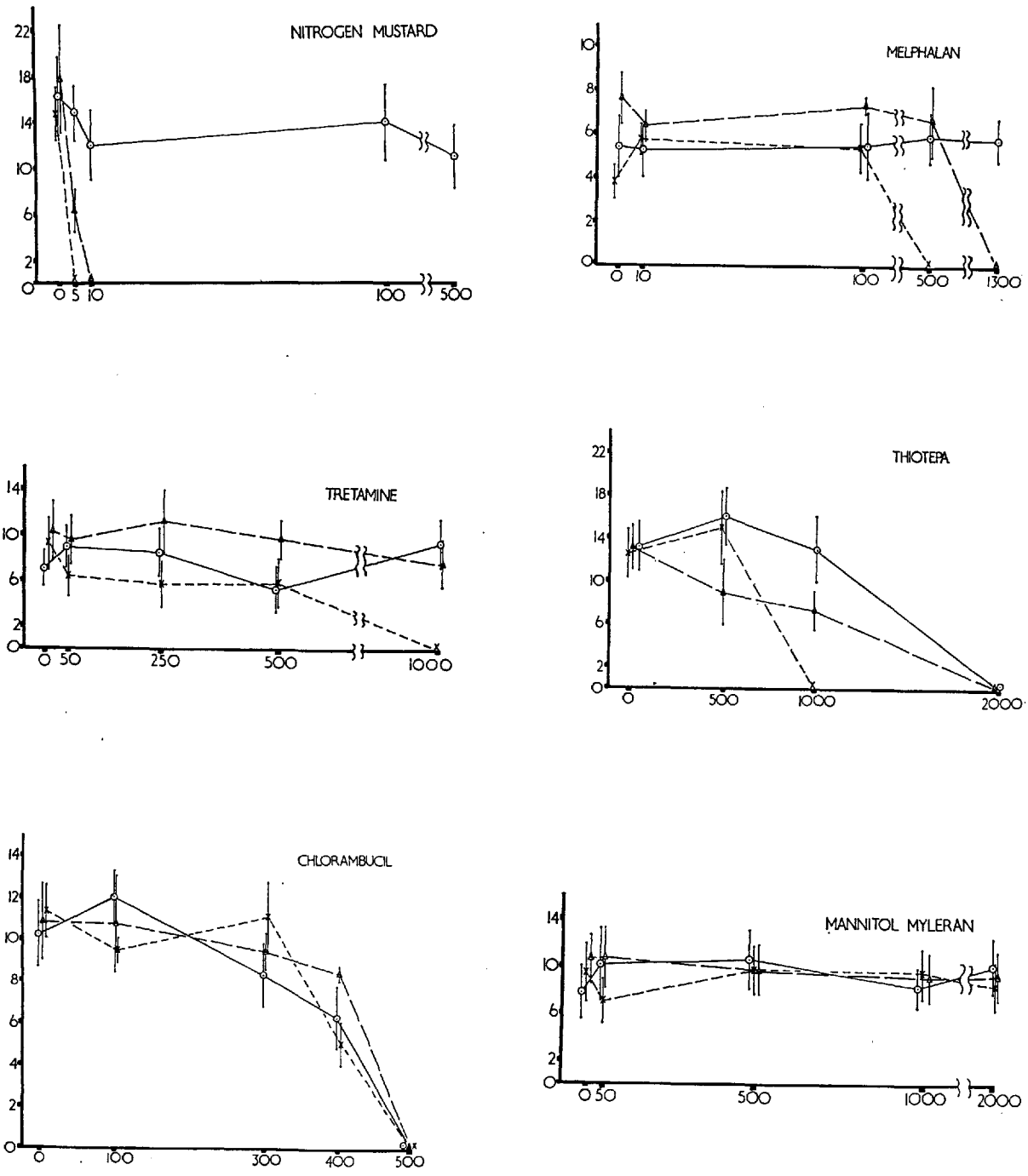
Treating the cells with drug before the addition of thymidine tended to decrease the uptake of thymidine by individual cells and reduce the proportion of cells labelled. In most cases, the longer the pretreatment time the lower the grain count at each drug concentration. The extent of this pretreatment effect depended on the drug. It was pronounced with nitrogen mustard and melphalan, moderate with thioTEPA and tretamine, and absent with chlorambucil. Five hundred microgrammes nitrogen mustard per 3×10^6 cells added immediately before the thymidine did not prevent thymidine uptake, whereas cells pretreated with as little as 10μg nitrogen mustard per 3×10^6 cells for 45 min showed no detectable uptake. After 1½ hr pretreatment, 5μg was sufficient to produce the same result. In contrast, cells incubated with chlorambucil incorporated the same quantity of thymidine whether the cells were incubated with drug and thymidine together or with thymidine added 45 min or 1½ hr after the drug.

Figs. 1–6. *Effect of selected alkylating agents on uptake of thymidine–H³ by mouse thymocytes.*



(A. Effect on grains per labelled cell.)
Grains/labelled cell: Ordinate
μg drug/3 × 10⁶ cells: Abscissa
⊙———⊙ no pretreatment
△—— ——△ ¾-hr pretreatment
×— — — —× 1½-hr pretreatment
•———• standard deviation

Figs. 7–12. Effect of selected alkylating agents on uptake of thymidine–H^3 by mouse thymocytes.



(B. Effect on percentage cells labelled.)

% cells labelled: Ordinate

µg drug/3 × 10^6 cells: Abscissa

⊙———⊙ no pretreatment

△—— ——△ ¾-hr pretreatment

×— — — —× 1½-hr pretreatment

•———• standard deviation

Table 1. *Drug concentrations (mM) causing "complete inhibition" and 50% reduction of thymidine uptake by mouse thymocytes*

Drug and pretreatment time (hr)	Complete inhibition		50% reduction	
	Based on results from grains/ labelled cell	Based on results from % cells labelled	In grains/ labelled cell	In % cells labelled
Nitrogen mustard				
0	>2·16	>2·16	0·13	>2·16
¾	>0·026 <0·043	>0·026 <0·043	0·015	0·013
1½	0·022	0·022	0·0087	0·0087
Tretamine				
0	>4·08	>4·08	3·77	>4·08
¾	>4·49 <5·51	>4·08	3·12	>4·08
1½	>2·65 <4·08	>2·45 <4·08	2·14	2·77
Mannitol myleran				
0	>4·93	>4·93	>4·93	>4·93
¾	>4·93	>4·93	>4·93	>4·93
1½	>4·93	>4·93	>4·93	>4·93
ThioTEPA				
0	>5·28 <8·81	>4·84 <8·81	5·88	5·64
¾	>4·62 <5·28	>5·06 <8·81	4·12	4·87
1½	>2·64 <4·40	>2·64 <4·40	3·57	3·41
Chlorambucil				
0	>1·23 <1·37	>1·15 <1·37	1·12	1·15
¾	>1·23 <1·37	>1·19 <1·37	0·68	1·19
1½	>1·23 <1·37	>1·15 <1·37	1·13	1·07
Melphalan				
0	>3·41	>3·41	>3·41	>3·41
¾	>1·70 <3·41	>1·55 <3·41	1·51	2·11
1½	>0·66 <1·31	>0·41 <1·31	0·83	0·73

Relative drug effectiveness

The probable molar concentration of each drug causing complete inhibition of detectable thymidine uptake, and the molar concentration of each drug causing a 50% reduction in the mean grain count per labelled cell and in the proportion of labelled cells from the control values, were judged from Figs. 1–12 (Table 1). Complete inhibition was said to have occurred when there were no more than four grains/cell. From these figures the drugs were placed in the order of their ability to interfere with thymidine uptake (Table 2). It was found that the order of effectiveness depended on which pretreatment time was selected. For example, with no pretreatment, chlorambucil was the most effective drug in preventing detectable uptake of thymidine. With 45 min pretreatment, nitrogen mustard was the most effective, with chlorambucil taking second place, and with 1½ hr pretreatment melphalan replaced chlorambucil as the second most active drug.

In most cases, the drug order obtained from reductions in uptake per labelled cell was the same as that obtained from reductions in the percentages of cells labelled. The one exception to this occurred when there was no pretreatment and 50% reduction from the control values was used as the end-point. In this case, chlorambucil was the most effective agent in reducing the proportion of labelled cells, but nitrogen mustard was the most effective in reducing the grain count per labelled cell.

DISCUSSION

It has been shown above that cell damage by these alkylating agents can be measured *in vitro*. Excepting mannitol myleran, all the alkylating agents examined decreased incorporation of exogenous thymidine into DNA of mouse thymocytes. Several drug actions could have caused this inhibition. Decreased thymidine uptake would occur: —

(*a*) if the thymidine to DNA pathway were directly blocked, or

(*b*) if damage took place before entry into the S phase, the thymidine to DNA pathway itself being undamaged, or

(*c*) if some of the cells were dead when the thymidine was added.

Each of these possibilities will be discussed in turn, with special reference to the present results and to the existing theories on the mode of action of alkylating agents.

a. *Direct inhibition of nucleic acid synthesis*

Inhibition of DNA synthesis by nitrogen mustard has been reported in many cell systems [for examples, see 11–18]. Some authors state that nitrogen mustard acts specifically on DNA synthesis, while others suggest that RNA synthesis is also affected and that protein synthesis may be reduced. These conclusions were reached with a variety of cell systems, both *in vivo* and *in vitro*, and with widely varying drug concentrations. There is some indication that the extent of the inhibitory action of nitrogen mustard depends on the drug concentration used. When Earle's 'L' cells were treated *in vitro* with 0·1μg / ml, DNA synthesis stopped 24 hr after drug treatment when the premitotic DNA level had been reached. RNA synthesis and protein synthesis were unaffected at this concentration but when the drug concentration was increased to 0·2–0·3μg / ml RNA synthesis and protein synthesis were also depressed and the cells eventually lysed [15]. Similarly, treating rabbits *in vivo* with 1·5–5 mg / kg nitrogen mustard and treating Earle's 'L' cells *in vitro* with 0·25μg / ml resulted in DNA synthesis being depressed more than RNA synthesis, whereas treating the more resistant Ehrlich ascites tumour cells *in vitro* with 50μg / ml resulted in both RNA synthesis and DNA synthesis being reduced to about 30–40% of the control values [19]. Some of the differences in drug action on metabolic pathways recorded by various authors can probably be explained in terms of differences in drug concentration used to treat tissues with different sensitivities to nitrogen mustard.

It has been shown that nitrogen mustard does inhibit nucleic acid synthesis in some cell systems, including the mouse thymocytes used in the present experiment. The mode of action of inhibition of nucleic acid synthesis by this drug is, however, disputed and a pretreatment effect would be compatible either with the idea that nitrogen mustard acts specifically on certain site(s) or with the idea that any reactive site may be attacked non-specifically [20, 21].

Table 2. *Order of drug effectiveness**

End-point	Pretreatment time (hr)	Based on results for per cent cells labelled	Based on results for grains per labelled cell
Complete inhibition of detectable thymidine uptake	0	C>others	C>others
	¾	N>C>Mp>$\frac{\text{Tr}}{\text{Th}}$	N>C>Mp>$\frac{\text{Tr}}{\text{Th}}$
	1½	N>Mp≥C>$\frac{\text{Tr}}{\text{Th}}$	N>Mp≥C>$\frac{\text{Tr}}{\text{Th}}$
50% reduction from control value	0	C>others	N>C>others
	¾	N>C>Mp>$\frac{\text{Tr}}{\text{Th}}$	N>C>Mp>Tr>Th
	1½	N>Mp>C>Tr>Th	N>Mp>C>Tr>Th

*Judged in terms of the molar concentration needed to produce the desired effect.

C — chlorambucil.

N — nitrogen mustard.

Mp — melphalan.

Tr — tretamine

Th — thioTEPA.

b. *Indirect inhibition of DNA synthesis resulting from a block in the cell cycle*

Any block in the cell cycle which prevented cells entering the synthetic phase (S phase) would eventually cause inhibition of thymidine uptake. Growth inhibition is not always associated with an immediate block in DNA synthesis. Thus, Ultmann *et al.* [22] obtained inhibition of mitosis in regenerating rat liver with doses of nitrogen mustard (1·9 mg/kg) which allowed the accumulation of both RNA and DNA beyond the control values. These increased values were thought to be due to the entry of fewer cells into the first mitosis after hepatectomy and, therefore, to an accumulation of cells with the premitotic DNA level. Similarly, growth inhibition of mouse tumours *in vivo* was produced with doses of nitrogen mustard (0·8 mg/kg) which had no inhibitory action on the incorporation of P^{32} into DNA [23]. Continued DNA synthesis up to premitotic levels in the presence of nitrogen mustard was also shown by Levis *et al.* [24] and by Brewer *et al.* [15]. These results indicate that nitrogen mustard can act in such a manner that DNA synthesis occurs normally up to premitotic levels but that further DNA synthesis is prevented, perhaps because the cells fail to divide.

Widner *et al.* [25], basing their arguments on the decrease in the mitotic index of transplanted rat tumours at various times after the rats were treated with nitrogen mustard, suggested several blocks in the cell cycle. It is generally accepted that nitrogen mustard acts during interphase though there are a few papers besides Widner's which suggest inhibition during metaphase (see Gelfant [26] for discussion and references). A block in the premitotic resting phase (G_2) is suggested by Layde and Baserga [27] and by Gelfant [26]. It is thought that cells that are in the early part of the G_2 phase when they are exposed to nitrogen mustard do not undergo mitosis. Cells in the latter part of the G_2 phase at that time are presumed to divide and pass through the following G_1 and S phases before being affected by the nitrogen mustard. This would explain the increase in dry mass [28] and the doubling of DNA content reported in cells treated with nitrogen mustard. RNA synthesis and protein synthesis are thought to be relatively unaffected.

If a G_2 block were taking place in the present experiment, a pretreatment effect would be expected. The longer the cells were in contact with the nitrogen mustard the greater the number of mitoses prevented and the fewer the cells entering the S phase. A lower percentage of tritium labelled cells would then result. To cause complete inhibition of thymidine uptake, the drug would need to be in contact with the cells for a pretreatment period equal to, or exceeding, the duration of the mitotic phase plus G_1 plus S plus any G_2 remaining after the block. Metcalf D. (personal communication) finds the cell cycles of large and medium thymocytes to be 6·8 and 8·2 hr respectively, with the S phases alone lasting 5·5 hr. The cell cycle of small thymocytes is much longer. Since a pretreatment period of 45 min with nitrogen mustard prevents detectable uptake of thymidine, the main action of nitrogen mustard on these cells cannot be a block in the premitotic resting phase. Moreover, appropriate concentrations of thioTEPA and chlorambucil prevented detectable thymidine uptake without pretreatment. The cells must have been damaged in the few minutes between the additions of drug and labelled precursor. The main action of these drugs under these experimental conditions cannot have been a block in the premitotic resting phase. The effect of nitrogen mustard has already been shown to depend on the concentrations used, and it is possible that with lower drug concentrations and longer pretreatment times such a G_2 block would be detectable in the present cell system.

A block in some part of the cell cycle other than the G_2 phase could result in a pretreatment effect but, again, complete inhibition of thymidine uptake would not be expected until a pretreatment time approaching the length of the cell cycle was used.

c. *Cell damage unrelated to DNA synthesis*

The cells could have been damaged at some site or sites not directly connected with thymidine uptake. Inhibition of thymidine uptake could have reflected cell death due to some unrelated damage. Using a partially synchronized cell suspension of Earle's 'L' cells, Walker and Helleiner [29] studied the effect of nitrogen mustard on the viability of the different stages of the cell cycle. The synthetic phase was found to be the most susceptible. Assuming that this finding is not an artefact resulting from the chemical synchronization of the cells and that it can be applied to other cell systems, then it is possible that the mouse thymocytes in the S phase at the time of drug treatment were being preferentially killed. Reduced grain counts and lower percentages

of labelled cells resulting from increasing the pretreatment time with nitrogen mustard before the addition of thymidine would be compatible with this idea. The alkylating agents with the most pronounced pretreatment effects would presumably be those with the greatest damaging ability.

In conclusion, from observations of the pretreatment times needed to prevent detectable thymidine uptake, it appears that the main mode of action of these alkylating agents cannot have been a block in the cell cycle. Direct interference with the thymidine to DNA pathway (possibly by attack on the various enzymes involved) or non-specific interference with various metabolic processes with resulting cell death are suggested as possible main actions of these drugs on the mouse thymocytes in the present experiment.

Pretreatment effect

Some of the alkylating agents examined have been shown to have a more pronounced pretreatment effect (nitrogen mustard and melphalan) than others (tretamine and thioTEPA). No pretreatment effect was found with chlorambucil. Two possible causes of the pretreatment effect are postulated:

(1) It could be due to a time lag between the damage and the demonstrable effect on DNA synthesis

(2) It could reflect continuous and cumulative damage as long as the cell is in contact with the drug. These two possibilities are being examined.

Although the compounds studied are all alkylating agents, they are distinct chemical entities and as such cannot be assumed to react in a uniform manner. It is possible that the pretreatment effect simply reflects the time needed for sufficient drug to react and that this time varies from one drug to another. Published data on reaction rates are mostly derived from the study of isolated cell constituents in non-biological media. Under such conditions, competition by other nucleophilic substances and interference by cellular membranes, which would occur in intact cells, are absent. Further, the extent of drug adsorption to proteins in the present experiment is unknown. Israels and Linford [30] have shown that individual proteins have their own characteristic binding abilities and reaction rates with chlorambucil. Moreover, the chlorambucil results are difficult to explain in terms of relative rates of reaction since the drug appears to be as effective when added a few minutes before the thymidine as after 1½ hr pretreatment. Perhaps the chlorambucil is not acting as an alkylating agent in this system but prevents entry of thymidine by altering the cell surface. It may be significant that the compounds with the extreme effects, nitrogen mustard and chlorambucil, carry opposite charges when in solution, the ethyleneimmonium ion resulting from cyclization of nitrogen mustard acting as a cation and chlorambucil acting as an anion. Melphalan carries both a cationic and an anionic group and the compounds with an intermediate pretreatment effect are neutral in solution.

The possibility that the alkylating agents combine directly with the tritiated thymidine has also been considered [31, 32] but alkylation of thymidine is unlikely as sites of greater reactivity are available [33] and unpublished results [C. A. Brown] also appear to contradict this idea.

Mannitol myleran

The fact that mannitol myleran failed to show any inhibitory action on thymidine uptake, even at a concentration of 2 mg/ml, requires comment. Kellner and Németh [34] report that 1 mg mannitol myleran/ml had a marked *in vitro* effect after 24 hr on fibroblasts of chick embryo hearts, fresh Crocker sarcoma and HeLa cells. Two mg/ml caused cell destruction. Easty and Wylie [35] found that as low a concentration as 2·5–250 µg/ml had a cytotoxic effect on glioma cells. On the other hand, 33·8 mg/ml was found by Onesti and Woodliff [5] to have no effect on oxygen-uptake by chronic granulocytic leukaemia cells *in vitro*, and Reid [36] showed that 0·5 mg/ml had no effect on the *in vitro* synthesis of uridine nucleotides and glycogen by rat liver. This would indicate that different tissues have very different sensitivities to mannitol myleran and that mouse thymocytes may be among those that are relatively resistant.

Practical applications

The object of this study is to investigate an *in vitro* method which, it is hoped, will yield results reflecting the effects of drugs on growth of individual tumours *in vivo*. In the practical application of such a test, it is irrelevant whether the drugs prevent DNA synthesis directly or indirectly.

It has been shown that the order of effectiveness of different drugs appears to depend on the length of pretreatment and, in some circumstances, on the criterion used to measure the drug effect. Which, if any, of these timing procedures and criteria would yield results reflecting the effects of the drugs on the same cell system *in vivo* cannot be decided *a priori*. This can be determined only by performing parallel *in vitro* and *in vivo* studies. A further complication in the application of this test is that the majority of human tumour cells have a cell cycle longer than that of mouse thymocytes. Longer incorporation times would be needed to yield a countable number of cells labelled with thymidine.

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RÉSUMÉ

Dans le but de tester une méthode de mesure de la sensibilité individuelle des tumeurs aux agents chimiothérapiques in vitro, *l'auteur a étudié l'action de divers agents alkylants sur l'incorporation* in vitro *de la thymidine tritiée dans les thymocytes de souris. Des concentrations appropriées d'ypérite azotée, de tétramine, de thioTEPA, de chlorambucil et de melphalan diminuaient le nombre moyen de grains par cellule marquée et le pourcentage de cellules marquées, tandis que le mannitol-myleran* (4,9 *mM*) *n'avait aucun effet sur l'incorporation de la thymidine. En augmentant la durée d'incubation avec le produit, avant l'addition de thymidine, on intensifiait l'action du médicament, mais l'importance de cette intensification variait d'une substance à l'autre. Cet accroissement d'effet, en fonction de la durée d'incubation, était importante pour l'ypérite azotée et le melphalan, modérée pour la tétramine et le thioTEPA, et absente pour le chlorambucil. L'efficacité apparente des substances (déterminée par leur faculté de diminuer l'incorporation de thymidine par rapport aux contrôles) dépendait donc de la durée de l'incubation des cellules avec les agents chimiothérapiques.*

A partir de ces observations, l'auteur discute la validité des méthodes de mesure de la sensibilité des tumeurs in vitro, *comme moyen d'évaluer la sensibilité de ces mêmes tumeurs* in vivo. *Des études parallèles* in vitro *et* in vivo *sont nécessaires pour déterminer quelle méthode est capable d'apporter des renseignements valables pour la clinique. On pourrait aussi déterminer, de cette façon, s'il est préférable d'utiliser le pourcentage de cellules marquées ou le nombre de grains par cellule, comme critère d'évaluation.*

Des observations sur l'importance du temps d'incubation préalable avec la substance (aucune préincubation n'était nécessaire pour empêcher l'incorporation de thymidine avec le chlorambucil et le thioTEPA; 45 *minutes de préincubation étaient nécessaires pour l'ypérite azotée et le melphalan; et une heure et demie pour la tétramine), on peut conclure qu'un arrêt, à un stade quelconque du cycle mitotique de la cellule, ne constitue pas le mode d'action principal de ces substances, dans les conditions expérimentales utilisées. Une interférence directe dans le processus métabolique conduisant de la thymidine à l'ADN, ou une interférence non spécifique dans divers processus conduisant à la mort de la cellule, sont proposées comme une explication possible du mécanisme d'action de ces substances.*

SUMMARY

With a view to examining a proposed method for measuring in vitro *the sensitivity of individual tumours to chemotherapeutic agents, the effects of selected alkylating agents on uptake of tritiated thymidine by mouse thymocytes* in vitro *were investigated. Appropriate concentrations of nitrogen mustard, tretamine, thioTEPA, chlorambucil and melphalan decreased the mean number of grains per labelled cell and reduced the percentage of labelled cells, while mannitol myleran* (4·9 *m M*) *had no effect on thymidine uptake. Increasing the incubation time with drug before the addition of thymidine tended to enhance the drug action, but the extent of the pretreatment effect varied from one drug to another. The pretreatment effect was pronounced with nitrogen mustard and melphalan,*

moderate with tretamine and thioTEPA, and absent with chlorambucil. As a result of the pretreatment effect, the apparent effectiveness of the drugs (determined by their ability to reduce thymidine uptake relative to control values) depended on the pretreatment time used.

The application of these findings to an in vitro *method of determining sensitivity of individual tumours* in vivo *is discussed. Parallel* in vivo *and* in vitro *studies are needed to indicate which, if any, of these incubation procedures and methods of reading the results would be of value in a clinical assay and, to some extent, on whether percentage cells incorporating thymidine or uptake per labelled cell should be used as the criterion of drug effect.*

From observations on the pretreatment time needed to prevent detectable uptake of thymidine (no pretreatment necessary with chlorambucil and thioTEPA, 45 min needed with nitrogen mustard and melphalan and 1½ hr with tretamine), it is concluded that a block at any one point of the cell cycle cannot be the main mode of action of these drugs under these experimental conditions. Direct interference with the thymidine to DNA pathway or non-specific interference with various metabolic processes resulting in cell death are suggested as possible modes of action of these drugs.

ZUSAMMENFASSUNG

Um eine vorgeschlagene Methode, welche dazu bestimmt ist die Empfindlichkeit einzelner Tumoren verschiedenen chemotherapeutischen Mitteln gegenüber in vitro zu untersuchen, zu überprüfen, wurde der Einfluss dieser Mittel aufddie Einverleibung von mit Tritium markiertem Thymidin in Mäusethymozyten in vitro studiert. Entsprechende Mengen von N-Lost, Tretamin, ThioTEPA, Chlorambucil und Melphalan führten zu einer Verminderung der Zahl der Körner pro markierte Zelle und des Prozentsatzes der markierten Zellen, während Mannitol-Myleran (4,9 mM) keinen Einfluss auf die Thymidineinverleibung hatte. Die Verlängerung der Inkubationszeit mit den Chemotherapeutica, ehe Thymidin hinzugegeben wurde, erhöhte die Wirksamkeit der letzteren, aber diese Wirksamkeit war uneinheitlich. Die Wirksamkeit dieser Vorbehandlung war sehr ausgesprochen im Falle von N-Lost und Melphalan, mässig mit Tretamin und ThioTEPA und abwesend mit Chlorambucil. Das Resultat der Vorbehandlung lässt den Schluss zu, dass die scheinbare Wirksamkeit, gemessen an ihrer Fähigkeit die Thymidineinverleibung im Vergleich zu den Kontrollwerten zu vermindern, von der Dauer der Vorbehandlung abhängt.

Die Anwendung dieser in vitro-Methode der Bestimmung der Sensibilität individueller Tumoren in vivo wird besprochen. Parallele Untersuchungen in vivo und in vitro sind notwendig um festzustellen, ob diese Inkubationsmethoden und die Anwendung ihrer Resultate klinisch verwendbar sind und zwar insbesondere um festzustellen, ob der Prozentsatz der Zellen, welche Thymidin einverleiben, oder ob die Einverleibung pro markierte Zelle als Prüfstein für die Wirksamkeit eines Medikamentes verwendet werden soll.

Aus der Bestimmung der notwendigen Vorbehandlungszeit, um eine erkennbar Thymidineinverleibung zu verhindern (mit Chlorambucil und ThioTEPA ist keine Vorbehandlung notwendig, mit N-Lost und Melphalan 45 Minuten und mit Tretamin 1½ Studnen) wird der Schluss gezogen, dass unter den genannten Versuchsbedingungen eine einfache Blockierung an irgendeinem Punkte des Zellteilungszyklusses nicht die Hauptwirkung dieser Medikamente ist. Die direkte Beeinträchtigung des Thymidin-DNA-Weges oder unspezifische Eingriffe in den Zellstoffwechsel, welche zu dem Zelltode führen, werden als mögliche Wirkungsmechanismen dieser Mittel in Erwägung gezogen.

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