Western University
Scholarship@Western

Digitized Theses

Digitized Special Collections

1974

Alkylation Carcinogenesis--a Comparative Study Of N-methyl-n-nitrosourea And Methyl-methanesulfonate

Subhas Chandra Maitra

Follow this and additional works at: https://ir.lib.uwo.ca/digitizedtheses

Recommended Citation
Maitra, Subhas Chandra, "Alkylation Carcinogenesis--a Comparative Study Of N-methyl-n-nitrosourea And Methyl-methanesulfonate" (1974). *Digitized Theses*. 739.
https://ir.lib.uwo.ca/digitizedtheses/739

This Dissertation is brought to you for free and open access by the Digitized Special Collections at Scholarship@Western. It has been accepted for inclusion in Digitized Theses by an authorized administrator of Scholarship@Western. For more information, please contact tadam@uwo.ca, wlswadmin@uwo.ca.

ALKYLATION CARCINOGENESIS - A COMPARATIVE STUDY OF N-METHYL-N-NITROSOUREA AND METHYL METHANESULPHONATE

by

Subhas Chandra Maitra, MBBS, DCP, MSc.

Department of Pathology

Submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

Faculty of Graduate Studies

The University of Western Ontario

London, Canada

1973

© Subhas Chandra Maitra, MBBS, DCP, MSc.

ABSTRACT

Earlier studies on the carcinogenic effects of monofunctional alkylating agents in Swiss mice have shown that a single intraperitoneal injection of methylnitrosourea induces thymic lymphomas, pulmonary adenomas and various other tumours; the yield of tumours has been seen to increase if it is given in split doses. It has also been observed that methylnitrosourea, after the tumour-inducing dose, causes temporary thymolymphoid and myeloid degeneration which is followed by rapid recovery of the tissues. A single dose of methyl methanesulphonate, on the other hand, has not been shown to induce tumours.

The present study was undertaken to isolate the causal events in the genesis of thymic lymphomas and pulmonary adenomas in Swiss mice. For this purpose, the two agents, methylnitrosourea and methyl methanesulphonate, were compared from three points of view: their ability to induce tumours, their ability to cause target tissue changes, and the nature of their reactions with DNA.

With regard to the first point, methyl methanesulphonate was tested in a sub-LD_{50} dose injected intraperitoneally; whether given in a single dose or in weekly doses for 5 weeks, it was found incapable of inducing tumours of any kind.

The second point was assessed by counting the number of nucleated cells in the bone marrow obtained from one full length femur and by noting the weights of thymus and of spleen at different times, e.g., 1,3,6,10,15, and 20 days, after a single intraperitoneal injection of a sub-LD_{50} dose of methyl methanesulphonate. Methyl methanesulphonate did not produce

any significant change in the bone marrow although it was found capable of reducing the weight of thymus. For better evaluation, three other agents, e.g., ethylnitrosourea, ethyl methanesulphonate and methylnitrosonitroguanidine, were also tested by the same criteria. Ethylnitrosourea, the only other agent known to induce thymic lymphomas, was able to cause both bone marrow and thymus changes.

The nature of the reactions with DNA was studied by isolating DNA from liver, gut, kidney, bone marrow, thymus, spleen and lung at different time intervals up to 12 hours following a single sub-LD_{50} dose of either (^{14}C)-methylnitrosourea or (^{14}C)-methyl methanesulphonate given intraperitoneally, hydrolysing the DNAs by a modified technique, separating the methylated purines by a new two-stage descending paper chromatography using a new solvent and detecting and quantitating the products by radioactive assay. The analyses revealed: greater generation of O^6-methylguanine in all tissues by methylnitrosourea: greater efficiency of alkylation by methylnitrosourea: significant tissue differences in the level of 7-methylguanine and possibly in the level of O^6-methylguanine following treatment with methylnitrosourea: preferential removal of 3-methyladenine over other methylated bases particularly in liver and kidney and relative lack of removal in bone marrow and spleen following treatment with methyl methanesulphonate and probably methylnitrosourea.

Both O^6-methylguanine and 7-methylguanine can induce a 'transition' mutation but the probability of such an event is much greater with O^6-methylguanine than with 7-methylguanine. Even then, the presence of O^6-methylguanine in tissues that do and others that do not develop tumours shows that it is not the only factor in neoplasia. An additional factor is the reparative hyperplasia in the target organs.

The other factors that may contribute to the induction of tumours by methylnitrosourea are: higher efficiency of alkylation compared to methyl methanesulphonate; slowing of DNA repair; and immunosuppression.

It would seem, therefore that multiple factors are involved in the genesis of methylnitrosourea-induced thymic lymphomas; the initial event is probably a permanent change in the DNA of target cells while the promotional stage is represented by tissue proliferation and immunosuppression.

ACKNOWLEDGEMENTS

It is difficult to convey in words the appreciation and gratitude I feel towards the persons who in different ways helped to make this conclusion possible.

I extend my most sincere thanks and gratitude to Dr. J. V. Frei, Associate Professor of Pathology, under whose guidance I carried on this study during the last two years. All through this period, it was my privilege to enjoy his encouragement, understanding and consideration.

My special gratitude is due to Dr. A. C. Wallace, Professor and Chairman of the Department of Pathology, for allocating the necessary space and for making all the facilities of his department available to me. I must not fail to express my appreciation for the sympathy and concern he had shown during the most perturbing personal problems which, at one time, had almost wrecked my morale.

My gratitude is also due to Dr. M. D. Haust, Professor of Pathology, for her encouragement during the first year of my training.

I am deeply indebted to Dr. I. G. Walker, Associate Professor of Biochemistry for his constructive criticism and helpful suggestions. My gratitude is due to him.

I was fortunate to have been able to get the advice and help of one of the pioneer researchers in the field of chemical carcinogenesis, Dr. P. D. Lawley of Chester Beatty Research Institute, England. I extend my sincere regard and gratitude to him.

Sincere thanks are also extended to Mrs. Jean Oliver for her skillful technical assistance.

I am deeply obliged to Dr. D. Bocking, Dean of the Faculty of Medicine, for providing me the financial support during the past two years.

LIST OF ABBREVIATIONS

A	Adenine
^{14}C	A radioactive isotope of carbon
DMN	Dimethylnitrosamine
DMS	Dimethyl sulphate
E_{260}	Extinction (absorption) of light in optical density units at the wavelength indicated in the subscript.
EMS	Ethyl methanesulphonate
ENUA	N-ethyl-N-nitrosourea
G	Guanine
MAM	Methylazoxymethanol acetate
MMS	Methyl methanesulphonate
MNNG	N-methyl-N-nitro-N-nitrosoguanidine
MNUA	N-methyl-N-nitrosourea
1-MeA	1-methyladenine
3MeA	3-methyladenine
7MeA	7-methyladenine
3MeC	3-methylcytosine
3MeG	3-methylguanine
7MeG	7-methylguanine
O^6MeG	O^6-methylguanine
T	Thymine

TABLE OF CONTENTS

CERTIFICATE OF EXAMINATION....ii

ABSTRACT....iii

ACKNOWLEDGEMENTS....vi

LIST OF ABBREVIATIONS....viii

TABLE OF CONTENTS....ix

LIST OF TABLES....xi

LIST OF FIGURES....xiii

I. INTRODUCTION AND PURPOSE OF THE THESIS....1

II. REVIEW OF LITERATURE....4

A. Early studies of chemical carcinogenesis....4

B. Suggested theories of chemical carcinogenesis....5

C. Alkylating agents....7

a. Groups, mechanisms, and biological effects....7

b. Cellular targets of alkylation and the possible modes of their action with respect to carcinogenesis....8

c. Handling of the damage....12

d. Immunosuppression....13

e. Virus activation....14

f. Tumour induction....15

g. Methyl-nitroso compounds....17

h. Methyl methanesulphonate....20

D. Historical aspects of lymphoma....23

E. Spontaneous lymphoma in mice....23

F. Induced lymphoma in mice....25

a. Radiation....25

b. Chemicals 25

c. Viruses 26

d. Hormones 27

e. Viruses in chemically and hormonally induced lymphomas 27

f. Combination of agents inducing lymphomas 27

G. DNA analysis 28

a. Isolation and purification 28

b. Hydrolysis 29

c. Separation of the products 30

III. EXPERIMENTAL WORK 34

A. Tumour induction by Methyl methanesulphonate 36

1. Materials and Methods 34

2. Results 35

B. Tissue changes following treatment with Methyl methanesulphonate, Ethyl methanesulphonate, Methylnitrosourea and Methyl-nitronitrosoguanidine 37

1. Materials and Methods 37

2. Results 38

C. Reaction products of DNA following treatment with ^{14}C-methylnitrosourea and ^{14}C-methyl-methanesulphonate 48

1. Materials and Methods 48

2. Results 62

IV. DISCUSSION 131

V. SUMMARY 175

BIBLIOGRAPHY 178

VITA 207

LIST OF TABLES

1. Carcinogenicity of MNUA....21
2. Carcinogenicity of MMS....24
3. Tumour induction by MMS and MNUA in Swiss mice....36
4. Tissue changes following administration of MMS, EMS, ENUA and MNNG in Swiss mice....40
5. DNA alkylation at 0.3 hour after MNUA....72
6. DNA alkylation at 1.0 hour after MNUA....73
7. DNA alkylation at 2.0 hours after MNUA....74
8. DNA alkylation at 4.5 hours after MNUA....75
9. DNA alkylation at 12 hours after MNUA....76
10. DNA alkylation at 0.3 hour after MMS....77
11. DNA alkylation at 1.0 hour after MMS....78
12. DNA alkylation at 2.0 hours after MMS....79
13. DNA alkylation at 4.5 hours after MMS....80
14. DNA alkylation at 12 hours after MMS....81
15. Balance sheet of cpm - DNA 0.3 hour after MNUA....82
16. Balance sheet of cpm - DNA 1.0 hour after MNUA....83
17. Balance sheet of cpm - DNA 2.0 hour after MNUA....84
18. Balance sheet of cpm - DNA 4.5 hours after MNUA....85
19. Balance sheet of cpm - DNA 12 hours after MNUA....86
20. Balance sheet of cpm - DNA 0.3 hour after MMS....87
21. Balance sheet of cpm - DNA 1.0 hour after MMS....88
22. Balance sheet of cpm - DNA 2,0 hour after MMS....89
23. Balance sheet of cpm - DNA 4.5 hours after MMS....90
24. Balance sheet of cpm - DNA 12 hours after MMS....91
25. Effect of various manipulations in the induction of thymic lymphomas in mice by x-rays....133

26. Comparison of analyses done by Dr. P. D. Lawley and the author - Gut DNA isolated 4.5 hours after MNUA and MMS....................139

27. Part - I: Total alkylation of DNA following MMS with mean, variance and standard deviation....................143

Part -II: Comparison of the means

28. Methylated purines in DNA in vitro or in vivo....................147

29. Comparison of analyses done by Dr. P. D. Lawley and the author - Gut and liver DNA isolated 4.5 hours after MNUA....................148

30. Methylated bases in RNA in vitro or in vivo....................149

31. Methylation products of DNA following MMS with mean, variance and standard deviation....................151

32. Methylation products of DNA following MNUA with mean, variance and standard deviation....................155

33. Part - I: Variance ratios MNUA/MMS (mean values)....................157

Part -II: Difference of means - MNUA vs MMS

34. Difference of means - peaks vs 0.3 hr. or 12 hrs or 1st and last values....................159

35. Ratio of alkylation products of DNA following MMS with mean, variance and standard deviation....................162

36. Ratio of alkylation products of DNA following MNUA with mean, variance and standard deviation....................163

37. Part - I: Variance ratios 3MeA/7MeG (mean values)....................164

Part - II: Comparison of means - 3MeA/7MeG ratio

Part - III: Comparison of means - O^6-MeG/7MeG ratio

38. Part - I: Total alkylation of DNA following MNUA with mean, variance and standard deviation....................172

Part - II: Comparison of the means

LIST OF FIGURES

1. Effect of MMS, EMS, ENUA and MNNG on bone marrow nucleated cell count 43
2. Effect of MMS, EMS, ENUA and MNNG on thymus weight 45
3. Effect of MMS, EMS, ENUA and MNNG on spleen weight 47
4. Two-stage radiochromatogram showing separation of metabolically labelled normal and methylated purines 65
5. Re-chromatogram of the zone corresponding to O^6-MeG showing its separation from the contaminants 67
6. Methylation of DNA in various mouse organs by MNUA 93
7. Percent of guanine methylated as 7MeG by MNUA 95
8. Percent of adenine methylated as 3MeA by MNUA 97
9. Percent of guanine methylated as O^6-MeG by MNUA 99
10. Proportion of 7MeG in different tissues relative to other identified methylated purines (after MNUA) 101
11. Proportion of 3MeA in different tissues relative to other identified methylated purines (after MNUA) 103
12. Proportion of O^6-MeG in different tissues relative to other identified methylated purines (after MNUA) 105
13. Metabolic incorporation of ^{14}C into guanine of DNA in different tissues after treatment with MNUA 107
14. Metabolic incorporation of ^{14}C into adenine of DNA in different tissues after treatment with MNUA 109
15. Ratio of metabolically labelled guanine/adenine in different tissues after treatment with MNUA 111
16. Methylation of DNA in various organs by MMS 113
17. Percent of guanine methylated as 7MeG by MMS 115
18. Percent of adenine methylated as 3MeA by MMS 117
19. Percent of guanine methylated as O^6-MeG by MMS 119
20. Proportion of 7MeG in different tissues relative to other identified methylated purines (after MMS) 121

21. Proportion of 3MeA in different tissues relative to other identified methylated purines (after MMS)....123

22. Metabolic incorporation of ^{14}C into guanine of DNA in different tissues after treatment with MMS....125

23. Metabolic incorporation of ^{14}C into adenine of DNA in different tissues after treatment with MMS....127

24. Ratio of metabolically labelled guanine/adenine in different tissues after treatment with MMS....129

25. Percent of unidentified radioactivity in the DNA of different tissues after treatment with ^{14}C-MNUA or ^{14}C-MMS....137

26. Radioactivity due to metabolically labelled guanine and adenine in different tissues after treatment with ^{14}C-MNUA and ^{14}C-MMS - expressed as percent of total known radioactivity....141

27. Percent of guanine methylated as 7MeG by MNUA in mouse liver-DNA (Preliminary data of Dr. J. V. Frei)....146

28. Mean values of different methylation products of DNA after treatment with ^{14}C-MNUA and ^{14}C-MMS $\pm$ s.d.....154

29. Ratios of 3MeA/7MeG and O^6-MeG/7MeG in different tissues after treatment with ^{14}C-MNUA and ^{14}C-MMS....166

The author of this thesis has granted The University of Western Ontario a non-exclusive license to reproduce and distribute copies of this thesis to users of Western Libraries. Copyright remains with the author.

Electronic theses and dissertations available in The University of Western Ontario's institutional repository (Scholarship@Western) are solely for the purpose of private study and research. They may not be copied or reproduced, except as permitted by copyright laws, without written authority of the copyright owner. Any commercial use or publication is strictly prohibited.

The original copyright license attesting to these terms and signed by the author of this thesis may be found in the original print version of the thesis, held by Western Libraries.

The thesis approval page signed by the examining committee may also be found in the original print version of the thesis held in Western Libraries.

Please contact Western Libraries for further information:
E-mail: libadmin@uwo.ca
Telephone: (519) 661-2111 Ext. 84796
Web site: http://www.lib.uwo.ca/

I. INTRODUCTION AND PURPOSE OF THE THESIS

Despite great efforts in cancer research, the progress towards an understanding of this major medical problem appears to be relatively slow. The quest for newer knowledge relevant to the problem continues unabated in different disciplines of biomedical sciences by individuals and by groups of workers employing a wide variety of inducing agents and experimental systems. It seems to be an obligation on all to try to offer helpful contributions on possible mechanisms of carcinogenesis in the hope that a true understanding will more rapidly emerge. It was in this spirit the present work was undertaken.

The historic discovery that cancer could be induced by pure chemicals of known structure (Kennaway, 1930) was greeted with much optimism. It was hoped that these chemicals, synthesized from radioactive materials and reacted with living cells, would quickly indicate the cellular targets responsible for carcinogenic transformation. Although the early promise of the discovery of chemical carcinogenesis has not been fulfilled, it has provided valuable information on the possible molecular mechanisms of carcinogenesis. Alkylating carcinogens are of particular interest since this group includes some of the chemically simplest agents. Methylation seems to be of further interest because of its simplicity and because biomethylation is a fundamental natural process. For those carcinogens that require metabolism in order to exert their biological effects, the reactive metabolites may also be alkylating agents (as with N,N-dialkylnitrosamines, Magee and Barnes, 1967), aralkylating agents (as with aromatic hydrocarbons, Dipple et al., 1968), or arylating agents (as with certain aromatic amines, Miller and Miller, 1966). Thus, for a wide range of

carcinogens, comparisons with simple alkylating agents may be useful in studying the mode of action. The alkylating agents seem also of special significance because some are found in the human environment or are produced following ingestion of certain foods and chemicals and may be causes of human tumours.

The present investigation was conducted in purebred Swiss-derived mice with two simple alkylating agents, methylnitrosourea and methyl methanesulphonate, because some basic experimental techniques were already established in this laboratory with this strain of mice, much information on the effects of this type of carcinogens in this experimental system was available (Frei, 1970; Joshi and Frei, 1970; Frei, 1971a, 1971b; Frei and Joshi, 1973), there was apparent tissue specificity of tumour induction of some of these agents (Frei, 1971a) which could be used as built-in controls in the study of their effects within the same animal, and because the pure-bred mice were available in large numbers. This background knowledge helped in designing the experiments and permitted omission of some experimental steps.

The carcinogenic effects of methylnitrosourea and methyl methanesulphonate were compared from three points of view: the first dealing with the induction of tumours, particularly thymic lymphomas, the second dealing with the changes in tissues believed to be involved in the development of thymic lymphomas e.g., bone marrow and thymus, and the third dealing with the nature of the reactions with DNA of tissues.

It should be mentioned here that many workers in this field of research use the term leukaemia and lymphoma interchangeably. Although leukaemia and lymphoma are related, the literature on spontaneous and induced neoplasms of the reticular system of the mouse (Gardner et al.,

1944; Moloney, 1959; Rappaport and Baroni, 1962; Metcalf, 1966b; Kaplan, 1967; Frei, 1970; Joshi, 1970) shows that the murine tumours originate in the thymus and may metastasize to the lymph nodes, spleen and bone marrow if they have not choked the mouse first by filling up its chest. Apparently only when bone marrow metastases have developed do neoplastic cells appear in the blood stream producing a true leukaemia. In this sense, a leukaemia is induced but it is not always present. These tumours should, therefore, be designated as thymic lymphomas. Other terminology that may appear in the review of the literature should be interpreted with these comments in mind.

The purpose of this thesis is to report the results of investigations which, it is hoped, have provided new and valuable information on the mechanism of carcinogenesis.

II. REVIEW OF LITERATURE

In an attempt to avoid repetition, the pertinent literature on alkylation carcinogenesis with respect to the development of thymic lymphomas in mice will be reviewed when the results of the appropriate experiments are discussed. The literature on other aspects relevant to the subject is reviewed below.

A. Early studies of chemical carcinogenesis

While the awareness of the existence of cancer as a disease goes back to the recording in an Egyptian papyrus of the fifteenth century B.C. (cited by Oberling, 1944), a cause and effect relationship between an environmental situation and a cancer resulting from it was first recorded only relatively recently. In 1775, Percival Pott, an English surgeon, made the remarkable observation of the prevalence of scrotal carcinoma in chimney sweeps and he attributed this to their occupational exposure to soot. In 1820, similar lesions were observed amongst workers in English factories which treated minerals containing arsenic and Ayrton Paris indicated the noxious nature of the vapours of this element. Towards the end of the nineteenth century and the start of the twentieth century many reports of human cancerous lesions from various professional origins were produced (quoted from Hueper, 1942: skin tumours in paraffin workers - Von Volkman, 1875; mule spinners' cancer of abdominal wall and of scrotum due to shale oil - Bell, 1876; bladder cancers among workers in the dye industry - Rehn, 1906). Attempts were also made, although unsuccessful, to induce cancers on animals by the application of chemicals

(quoted from Hueper, 1942: Hanau, 1889; Lubarsch, 1890; Cazin, 1894; Borsch, 1900; Stahr, 1907; Haga, 1913).

Between 1910 and 1914, successful induction of tumours in animals was achieved for the first time by three different means: by physical agents (X-rays; Marie, Clunet and Lapointe, 1912, quoting their report of 1910), chemical agents (tar: Yamagiwa and Ichikawa, 1917, quoting their 1914 report), and living agents (fowl tumour virus: Rous, 1910).

The initial success of Yamagiwa and Ichikawa with experimental chemical carcinogenesis was achieved by the repeated application of tar to rabbit's ears. In the following years, this fundamental discovery was confirmed and extended to other species of animals, in particular the mouse (Tsutsui, 1918). From 1920 to 1930, elaborate investigations were carried out by the English school and these led to the induction of skin tumours in mice upon application of a pure hydrocarbon 1,2,7,8-dibenzanthracene (Kennaway, 1930). Only five years later, the carcinogenic activity of the first of the visceral carcinogens, o-aminoazotoluene for the liver of rat, was reported by Sasaki and Yoshida (1935). Later Hueper and his associates (1938) demonstrated that 2-naphthylamine was carcinogenic for the urinary bladder of dogs. The succeeding years had seen studies on the mechanism of action of these and many other chemical carcinogens develop on a broad international basis.

B. Suggested theories of chemical carcinogenesis

The theories of chemical carcinogenesis may be grouped under four headings: the somatic mutation theory, the genetic regulation theory, the latent virus theory, and the immunological theory.

The concept that neoplasms arise from "mutations" in somatic cells has long been a popular theory of cancer causation (Boveri, 1914; Bauer, 1928, 1963). The modern understanding of the chemical basis of mutation has enhanced its acceptance as a satisfactory theory for the mechanism of action of chemical carcinogens, particularly since chemical carcinogens or their metabolites have been frequently found to have mutagenic properties (Clayson, 1962). Nevertheless, other theories compete strongly in directing experimental approaches on the genesis and nature of neoplasia.

Quasi-permanent and heritable changes in genome expression without changes in the genome itself provide another method for the development and perpetuation of clones of cells with altered growth characteristics (Jacob and Monod, 1961; Monod et al., 1963; Pitot and Heidelberger, 1963; Gelboin, 1967; Weinstein, 1969).

Viral activity as the basis for the origin of chemically induced neoplasia is also a popular idea since many carcinogenic viruses are now known. According to this concept, the chemical carcinogen activates a latent virus or causes the expression of a part of all of an integrated viral genome which can then induce neoplastic changes through either of the two previous mechanisms (Igel et al., 1969; Huebner, and Todaro, 1969; Temin, 1971).

The immunological theory suggests that chemical carcinogens alter the selection pressures in the cellular environment and thus permit the latent tumour cell clones to grow; this may be achieved either by changes in the antigenic pattern of cells (Green, 1954, 1958) or by immunosuppression (Prehn, 1963).

C. Alkylating agents

a) Groups, mechanisms, and biological effects: From the chemical point of view, the biological alkylating agents are a diverse group of organic compounds which commonly include: sulphur and nitrogen mustards; methanolamides; epoxides and ethyleneimines; lactones and related compounds; alkyl methanesulphonates; the nitroso compounds; aliphatic hydrazo, azo and azoxy compounds and triazenes; pyrrolizidine alkaloids; and ethionine and the halogenated hydrocarbons. All have the common property of alkylating a variety of sites in biological materials under physiological conditions.

Alkylation is the replacement of a hydrogen atom in a molecule by an alkyl radical or the addition of the radical to a molecule containing an atom in a lower valency state; the carbon atom through which the attachment is made becomes a fully saturated one. Agents that may act this way either per se or after conversion in vivo, are included in this class of compounds. In the alkylation process the donor molecule together with its electrons becomes more or less detached from the alkyl group and the positively charged radical- usually a carbonium ion - then seeks electrons e.g., reacts with nucleophilic centres. Depending upon the number of functional alkyl groups present, the agents may be monofunctional, bifunctional or polyfunctional. According to the concept attributable to Ingold (1953, 1970), two general types of reaction are described. In the first, SN_1, the driving force for the reaction is the solvent which dissociates the carbonium ion and the mechanism of alkylation is unimolecular. The carbonium ion is an extremely unstable and reactive species and is, therefore, indiscriminate in its combination with nucleophilic centres. In the second, SN_2, a complete separation of the carbonium ion does not occur

but a transition complex is formed involving both reactants and the substitution takes place in a bimolecular process. Under this concept, it may be deduced that if alkylating agents react through SN_1 mechanism, a wider spectrum of groups will be attacked than when the bimolecular SN_2 mechanism operates exclusively. More recent theories have abandoned the clear-cut distinction as outlined above, and attribute the difference between the various reagents to the relative rates of ion-pair formation and of the reaction of the ion-pair with the nucleophile (Sneen and Larsen, 1969). However, the general distinction between SN_1 and SN_2 types is still pertinent to the present discussion.

As a class the alkylating agents have generally proved to have potent cytotoxic and mutagenic activities; some of the agents have carcinogenic activity as well (Ross, 1962; Loveless, 1966).

b) Cellular targets of alkylation and the possible modes of their action with respect to carcinogenesis: Studies on the alkylation of cellular constituents *in vivo* indicate that DNA, RNA, and proteins are all alkylated to about the same extent as measured per unit weight (Brookes and Lawley, 1961). Although each of the three macromolecules has been postulated to be the significant target of action of chemical carcinogens, the recent rapproachment between the viral and chemical theories of carcinogenesis made possible by the discovery of RNA-dependent DNA polymerase in the oncornaviruses (Baltimore, 1970; Temin and Mizutani, 1970), has strengthened the view that the significant target of carcinogens is the genome as represented by DNA. The subsequent review will, therefore, deal mostly with the alkylation of DNA.

It has been postulated that the alkylating carcinogens react directly with the bases of DNA (Brookes and Lawley, 1964). The sites of alkylation of base residues in DNA in vitro and in vivo are now known to include: in guanine N-7, 0-6, and N-3; in adenine N-3, N-1, and N-7; and in cytosine N-3 (Lawley, 1972). The N-7 atom of guanine is the principal site of alkylation in all cases but the proportions of the various sites alkylated have been shown to differ significantly for the two types of agent, SN_1 and SN_2, in particular at the 0-6 atom of guanine (Lawley and Thatcher, 1970; Lawley et al., 1971; Lawley, 1972). Initially it appeared that the 0-6 atom of guanine is reactive only to the SN_1 agents (Loveless, 1969; Lawley and Thatcher, 1970; Lawley et al., 1971) but recently more refined analytical methods have revealed a low reactivity of the atom towards SN_2 agents (Lawley and Shah, 1972a).

Alkylation at N-7 atom of guanine moieties in DNA increases the acidity of the N-1 atom of the alkylated guanine so that the amount of the ionized form present at neutral pH will be increased in comparison with the normal base (Lawley and Brookes, 1961). When in this ionized state, the alkylated guanine can form a base pair with thymine involving formation of two hydrogen bonds and having the dimensions required by the Watson and Crick structure of DNA. The result of anomalous base pairing of this type during DNA replication would be to replace the affected G-C pair by an A-T pair and this error will be continued in subsequent replications. Wave mechanical calculations of the electronic distribution in the G-C pair also lead to the conclusion that a proton would tend to be lost from the N-1 atom of the alkylated guanine residue, but in addition it has been suggested that this proton is transferred to the complementary cytosine base, with the simultaneous transfer of a proton from the amino group of

cytosine to the extranuclear oxygen atom of guanine (Nagata et al., 1963). The difference between the predictions of these two hypotheses is that, as both bases of the alkylated G-C pair are thought to be changed to the anomalous tautomeric forms simultaneously, both the newly synthesized DNA strands would be changed, each giving new molecules having the original G-C replaced by the A-T base pair.

The second most reactive site of alkylation in DNA is at N-3 of adenine moieties (Lawley and Brookes, 1963). Neither the ionization nor the wave-mechanical theories suggest any definite molecular mechanism for anomalous base-pairing by the alkylated adenine.

The second mispairing base is the 0^6-alkylguanine (Loveless, 1969). It has been suggested that it could lead to atypical base-pairing because the N-1 position of guanine which is involved in the hydrogen bonding with cytosine would no longer bear a proton after 0^6-alkylation.

The third potential cause of anomalous base-pairing is that of the amino form of 3-alkylguanine (Lawley et al., 1971). This product has been shown to be formed in small amount by both SN_1 and SN_2 agents in vitro (Lawley et al., 1971) as well as in vivo (O'Connor et al., 1973). Since this base would probably pair normally in the imino form, and the predominant tautomeric form is not known, the possibility of its miscoding could be very low and may be even zero (Lawley, 1972).

There is also a strong evidence for miscoding when polynucleotide containing the modified base, 3-alkylcytosine (3-methylcytosine), is employed in polymerase system in vitro (Ludlum and Wilhelm, 1968; Ludlum, 1970, 1971). This product has been shown to be formed to about the same extent as 3-alkylguanine by both SN_1 and SN_2 agents in vitro and in vivo

(Lawley, 1972). It has been suggested (Ludlum and Wilhelm, 1968) that the introduction of a positive charge in the template in the region of alkylated base might alter local structure sufficiently to allow incorporation of a noncomplementary nucleotide.

The speculative anomaly in base-pairing can operate as long as the alkylated base remains attached to the DNA molecule. Alkylation of both guanine and adenine has been shown to result in slow hydrolysis of the products from the macromolecule (Lawley and Brookes, 1963). While this depurination may result in chain fission and thus in inactivation of the DNA, it is possible that it could result in mutation either due to a base pair deletion (Kreig, 1963; Corbett et al.,1970) or due to filling of the deficiency at random (Bautz - Freese, 1961). In the production of these point mutations, it might be expected that monofunctional alkylating agents would be more effective than the bifunctional agents.

In RNA, the reactive sites have been found to be the same as DNA and again the principal site of reaction is N-7 of guanine (Lawley, 1972). RNA can act as a template for RNA replications (Burdon and Smellie, 1962; Montagnier and Sanders, 1963) but the relevance of RNA directed RNA synthesis to the control of cell division is not known. The more significant function of RNA may be that of a repressor in genetic control of enzyme synthesis (Jacob and Monod, 1961) or the particular case of t-RNA as the repressor in the control of RNA synthesis (Kurland and Maaloe, 1962). Whether RNA acts in this way is uncertain since proteins are now being considered as more likely agents for this role (Monod et al., 1963; Pitot and Heidelberger, 1963). The more recent work by Axel et al, (1967) has stimulated reconsideration of the older concepts. It has been demonstrated that liver t-RNA obtained from rats fed on the hepato-carcinogen ethionine

is deficient in a minor leucine t-RNA and such loss of amino acid acceptance capacity is specific. Quite a few reports are now available showing that several carcinogens preferentially attack the t-RNA fraction of the target tissue (Farber, 1968; Ortwerth and Novelli, 1969; Agarwal and Weinstein, 1970). In the light of this evidence and the present concept that certain aspects of cell regulation and differentiation are normally exerted at the level of translation, rather than transcription (Weinstein, 1970), it is conceivable that RNA could be a significant cellular target.

Besides the possible role in the control of genetic expression, proteins could also be the significant cellular receptors from the point of view of the immunological theory of carcinogenesis. The alkylating agents could be carcinogens as a result of their ability to react with tissue-specific antigens (proteins) changing their specificities. There is experimental evidence that bifunctional alkylating agents can modify the immunological properties of proteins (Watkins and Wormall, 1952), although the sites of alkylation involved were not specified. Subsequent studies on the alkylation of proteins _in vitro_ and _in vivo_ have shown that the thiol group of glutathione is readily alkylated (review: Harington, 1967); others (Stein and Moore, 1946; Windmueller et al., 1959) have shown that alkylation of sulphur of methionine and the ring nitrogens of histidine also occurs. Whatever the site of alkylation, the extent of reaction with proteins within the cells is low and, unless considerable selectivity occurs, little biological damage might be expected.

c) Handling of the damage: Our present knowledge of molecular biology tells us that RNA or protein, if damaged, may be discarded because both macromolecules occur in multiple copies and can be regenerated from DNA. Hence no specific mechanisms of repair of alkylation damage of these polymers

are known, considered necessary or sought after.

DNA, in contrast, at least on the basis of genetic studies, is a unique molecule and when damaged may be irretrievably lost or changed; in either event the ultimate outcome would be detrimental to the cell unless some repair process operates. There is some evidence that some types of DNA may be represented by multiple identical copies but the significance of these observations is yet to be established (Britten and Davidson, 1969; Brown and Blackley, 1972; Medvedev, 1972).

The stimulus for the search of possible repair mechanism after chemical damage stemmed from the demonstration that the DNA damaged by either U.V. or X-ray irradiation (pyrimidine dimers and X-ray breaks) could be repaired by a "cut and patch" repair mechanism (Pettijohn and Hannawalt, 1964; Painter and Cleaver, 1967) and present evidence suggests that alkylation damage of DNA may be similarly repaired (Roberts et al., 1968; Hahn et al., 1968; Ayad et al., 1969; Lieberman et al., 1971; Fox and Ayad, 1971; Roberts et al., 1971; Roberts, 1972).

It may be anticipated that failure to repair or a faulty repair of alkylation damage of DNA could lead to either point mutations or segment mutations (Loveless, 1966).

d) Immunosuppression: Alkylating carcinogens are immunosuppressants (Malmgren et al., 1952; Parmiani et al., 1971; Gryschek, 1971) in common with other carcinogens (Prehn, 1963; Stjernsward, 1965, 1966, 1967; Salaman and Wedderbrown, 1966; Ball et al., 1966; Chan et al., 1968; Ceglowski and Friedman, 1968). While not all immunosuppressants are known to be carcinogenic, such immunosuppressants as corticosteroids have been shown to potentiate chemical carcinogenesis with methylcholanthrene (Sulzberger et

al., 1963) and to induce metastases of tumours that would not ordinarily metastasize (Agosin et al., 1952). In fact, all known immunosuppressants are known to foster the malignant adaptation (review: Gatti and Good, 1970). It is conceivable that a successful carcinogen should not only be capable of transforming its target cell, it should also effectively suppress or misdirect the host's immunologic mechanisms of surveillance and rejection against the possible new antigens of the transformed clone.

e) Virus activation: This phenomenon represents one of the most intriging aspects of tumour induction by alkylating agents and other chemical carcinogens. The present ideas of virus oncogenesis conceive that the viral genome remains integrated with the host genome (Dulbecco, 1965; Huebner and Todaro, 1969; Temin, 1971) analogously to the prophage of micro-organisms. It could be that any agent that would react with the genetic material would be capable of activating the virus. Bifunctional alkylating agents have been shown to be capable of releasing lysogenic phage in E.coli (Lwoff, 1953), and Endo et al. (1963) have shown that within a series of derivatives of 4-nitro-quinoline 1-oxide, this ability is closely correlated with carcinogenicity. Tumour-virus activity has also been shown after treatment with many other carcinogenic chemicals (Haran-Ghera, 1967; Zimmermans et al., 1969; Bergs et al., 1970; Ball and McCarter, 1971). Contrary reports are also available where visible virus activation could not be demonstrated after a tumour inducing dose of an alkylating carcinogen (Frei et al., 1973). Further, the putative tumour virus has been demostrated in normal tissues (Teitz et al., 1971; Ball and McCarter, 1971; Torre and Porta, 1972; Frei et al., 1973). These contradictory observations may lead to the redefining of the term "virus activation" as funct-

ional expression of the viral information. This need not mean numerical enrichment only. The nature of this activation, whether it means insertion of multiple copies of viral genome in the host genome, attachment of viral genome at specific sites of host genome, removal of viral repressor, or virus mutation, is not yet known, and no molecular mechanism for the process has yet been proposed.

f) Tumour induction: The reports of Boyland and Horning (1949), Heston (1949, 1953) and Griffin et al. (1951) of the induction of tumours (local sarcomas, lymphomas and pulmonary adenomas) in experimental animals (rats and mice) following subcutaneous or intravenous injections of a number of nitrogen mustards (HN2 and HN3) or following exposure to sulphur mustard initiated the interest in more elaborate experimental studies with other alkylating agents. Ethyleneimines and epoxides, lactones and related compounds, nitroso compounds, aliphatic hydrazo, azo and azoxy compounds and triazenes, pyrrolizidine alkaloids have since been explored for carcinogenicity in a more systematic way.

From studies of the carcinogenic action of a series of monofunctional ethyleneimines together with the polyfunctional compound triethylenemelamine (TEM), Walpole and his co-workers (1954) concluded that a number of compounds tested could induce sarcomas on injection, but the very much lower dose of TEM needed to produce a significant yield of tumours compared with the dose used with the monofunctional agents, emphasized the greater potency of the polyfunctional compound.

Haddow (1958) observed the induction of subcutaneous sarcoma in mouse, rat, and hamster on injection of the diepoxide, 1,2-3,4-diepoxybutane as well as by a number of bifunctional aromatic nitrogen mustards.

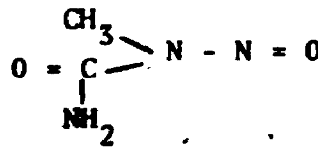
Van Duuren and co-workers (1963, 1965, 1966, 1967a, 1967b) studied the carcinogenic activity of various epoxides, lactones and peroxy compounds in mice and rats by both local application and subcutaneous injection. Whether locally applied or given subcutaneously only local tumours were observed. Dickens and Jones (1961) studied the activity of various lactones containing 4-, 5-, and 6-membered rings in rats by repeated subcutaneous injection. Although the 5-membered ring lactones showed higher activity than the 4-membered ring lactones only local tumours were induced and the investigators concluded that these compounds are slow acting carcinogens.

The carcinogenic activity of the hydrazo, azo, and azoxy compounds and triazenes was investigated by Preussmann et al. (1969) in rats. Hydrazomethane and azoxymethane on repeated subcutaneous or oral administration selectively produced carcinoma of the colon and rectum, whereas the corresponding ethyl compounds produced malignant tumours of the brain, the olfactory bulb, the breast and the liver and leukaemias but never intestinal carcinoma. 1-phenyl-3,3-dimethyl-triazene, after oral as well as subcutaneous injection induced malignant tumours of the brain and nervous system in BD rats. The carcinogenicity of certain triazenes was further demonstrated by the induction of malignant kidney tumours in single dose experiments.

The hepatocarcinogenic action of certain pyrrolizidine alkaloids was studied in chickens by Campbell (1956) and in rats by Schoental and Bensted (1963). A single oral dose was seen to produce hepatomas in the experimental animals.

The tumour inducing ability of the nitroso compounds was discovered by Magee and Barnes (1956). These compounds have been found to be extraordinarily active and almost all organs seem to be susceptible if the right compound is given (Druckrey et al., 1967; Reviews: Magee and Barnes, 1967; Magee and Swann, 1969; Magee, 1969). The range of animal species found to be susceptible to the induction of tumours is remarkably wide (Schmahl and Osswald, 1967) and includes the monkey (Kelly et al., 1966). These compounds have not only been shown to induce tumours after only one dose (Magee and Barnes, 1962; Druckrey et al., 1963) but have also shown remarkable organ specificity in their carcinogenic action (Druckrey et al., 1967). For example, when given in a single sub-lethal dose, dimethylnitrosamine induced kidney carcinomas, whereas when given in divided doses it induced liver carcinomas (Magee and Barnes, 1962); dibutylnitrosamine induced bladder cancer in the rat (Druckrey et al., 1964b); diethylnitrosamine induced tumours of the respiratory tract in hamsters (Herrold, 1964).

g) Methyl nitroso compounds: N-methyl-N-nitrosourea (MNUA), which was extensively used in this study, is such a compound and belongs to the subclass 'alkyl nitrosamides'. The structural formula is:



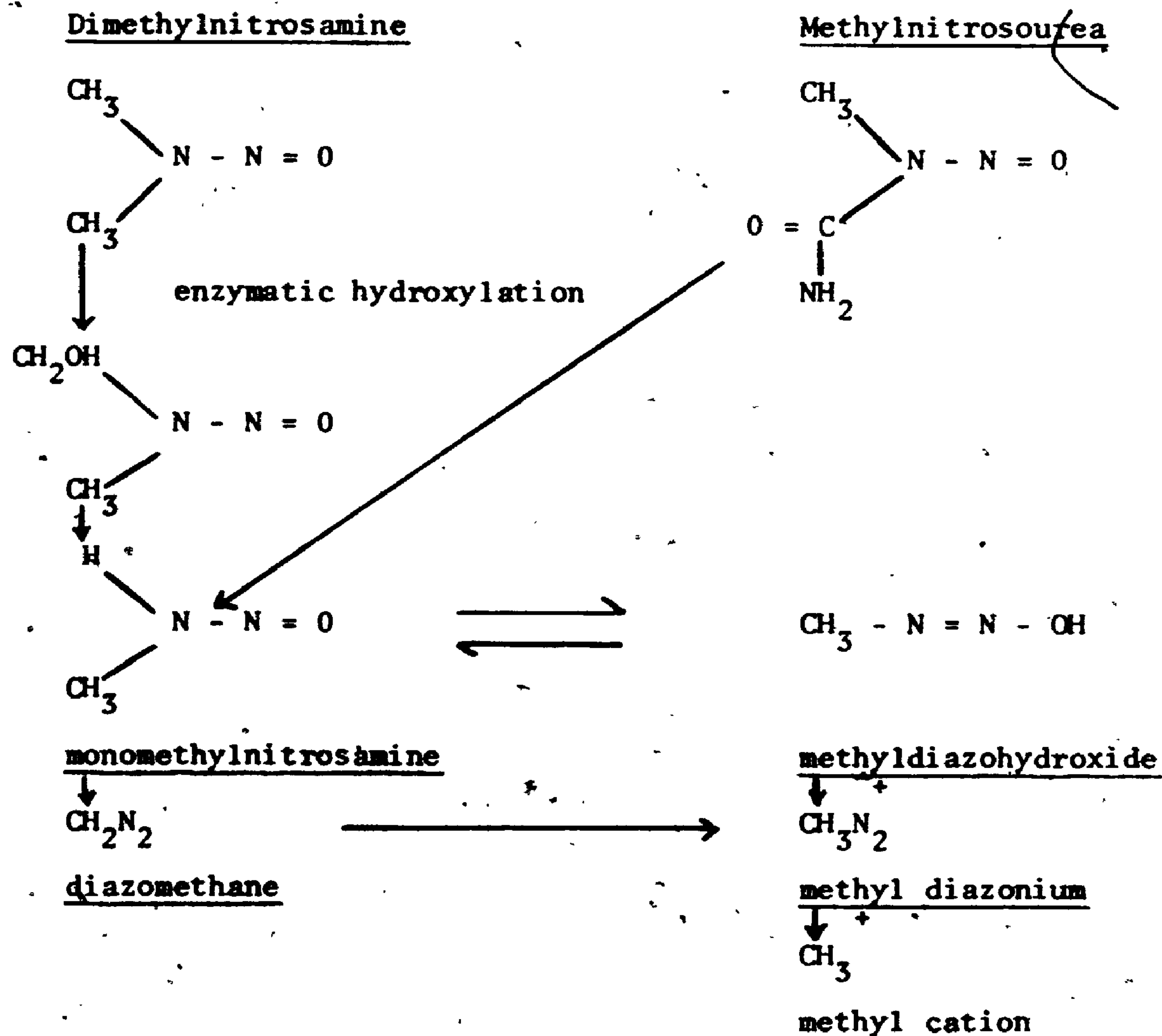
Nitroso compounds are photosensitive and MNUA is no exception to this generalization. From the study of photolysis of some of the nitroso compounds, it has been suggested that the nitroso group is split off as a radical and is rapidly converted into nitrous acid (McCalla et

al., 1968).

This compound is unstable in aqueous solution and according to Druckrey et al. (1967) its half life at 37°C is 125 hours at pH 4.0, 24 hours at pH 6.5, 1.2 hours at pH 7.0 and 0.1 hours at pH 8.0.

Although studies on the metabolism of MNUA in vivo have yet to be undertaken, the probable metabolic pathway has been suggested (Magee, 1968; Druckrey et al., 1969) on the basis of observations with dimethylnitrosamine (DMN). The metabolism of DMN was studied in rats, mice and rabbits by Magee (1956) using the polarographic method of Heath and Jarvis (1955) and by Dutton and Heath (1956) in the rat and the mouse using (^{14}C)-DMN. It has been suggested that the initial step is an enzymatic activation predominantly in the liver (Dutton and Heath, 1956; Magee, 1956; Magee and Vandekar, 1958) generating the reactive intermediate monomethylnitrosamine (Heath and Dutton, 1958) or methyl diazohydroxide (Austin, 1960). This may be supposed to react per se, or to ionize to give diazomethane, which may also react or further dissociate yielding the carbonium ion (Magee and Barnes, 1967). Magee and Barnes (1967) have proposed that diazomethane is the active agent in methylations by N-methyl-N-nitroso compounds, but Lijinsky and his associates (1968) have shown that with CD_3-labelled DMN in vivo the methyl group entered 7-methylguanine intact. Lingens et al. (1971) have reported analogous findings for methylnitrosonitroguanidine and DNA in E.coli. It seems likely that methylations by N-methyl-N-nitroso compounds, in general, are mediated by the methyl diazonium ion. With MNUA, the only dissimilarity suggested is the initial step of breakdown, which unlike DMN is simply chemical, and the same common alkylating species is produced. In any case, whatever may be the reactive form, MNUA

predominantly reacts through the SN_1 mechanism. The proposed metabolic pathways of DMN and MNUA are shown below:



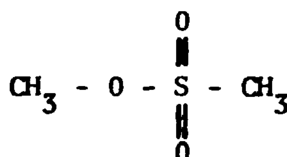
Recently, all N-alkyl-N-nitrosoureas have been reported to carbamoylate DNA bases besides alkylation (Serebryányi and Mnatsakanyan, 1972). The authors have suggested that these reactions might be significant in inducing mutations.

The other possible decomposition products of MNUA are: formaldehyde, nitrous acid, hydroxylamine derivative, hydrazine derivative (review: Magee and Barnes, 1967), and methanol (Swann, 1968). Except

formaldehyde and methanol all other decomposition products have, at times, been incriminated as potential carcinogenic forms.

MNUA is one of the most potent and versatile carcinogens. Its tumour inducing ability is shown in Table 1.

h) Methyl methanesulphonate (MMS): It is the methyl ester (primary) of methanesulphonic acid and has the structural formula:



It acts as a monofunctional alkylating agent because in reaction the alkyl-oxygen bond undergoes fission. It reacts by the SN_2 mechanism whereas the ethyl ester reacts by the mixed mechanism. For secondary esters the SN_1 mechanism predominates (Ross, 1962). Hudson (1959) has suggested that this variation is due to differences in the structure of the transition complexes.

Unlike MNUA, MMS is not remarkably sensitive to light or to pH; its half life at $37^{\circ}C$ is about 5 hours at pH 7.0 (Lawley and Thatcher, 1970).

Besides the release of the alkylating species and consequent alkylation of the cellular macromolecules, the other breakdown products of MMS are methanol (Pillinger et al., 1965; Cumming and Walton, 1970) and methanesulphonic acid (Cumming and Walton, 1970). Methanesulphonic acid in the concentrations usually involved in the biological experiments is rapidly and almost completely excreted unchanged in the urine (Trams et al., 1959), while the methanol is largely oxidized to CO_2 and excreted by the lungs (Pillinger et al., 1965; Cumming and Walton, 1970).

TABLE 1

Carcinogenicity of N-methyl-N-nitrosourea

Species	Organ	Treatment	References
Rat	Skin	Local applications	Graffi et al. (1967)
		Single intragastric dose in citrate buffer	Leaver et al. (1969)
		Single intraportal dose	Lijinsky et al. (1972)
	Stomach	A few oral doses	Druckrey et al. (1961)
		A few intragastric doses in phosphate saline	Schreiber and Jänisch (1967)
	Intestine	Single intragrastric dose in citrate buffer	Leaver et al. (1969)
		Single intravenous dose	Druckrey et al. (1963)
		Single intraportal dose	Lijinsky et al. (1972)
	Kidney	Single intragastric dose in citrate buffer	Leaver et al. (1969)
		Single intraportal dose	Lijinsky et al. (1972)
	Brain & Nerve tissue	A few oral doses in buffered drinking water	Thomas et al. (1967)
		Single intravenous dose	Janisch and Schreiber (1967)
		Repeated intravenous doses	Druckrey et al. (1964a, 1965, 1967)
			Stroobant and Brucher (1968)

Contd....

TABLE 1 Contd....

Species	Organ	Treatment	References
			Swenberg et al. (1972)
		Single intraportal dose	Lijinsky et al. (1972)
	Lymph node, Thymus, Liver, Oral Cavity, Testis, Uterus & Soft tissues	Single intraportal	Lijinsky et al. (1972)
Mouse	Skin	Local applications	Graffi et al. (1967)
	Lymphoma	Local applications	Graffi and Hoffmann (1966)
		Single subcutaneous dose	Terracini and Stramignoni (1967)
		Single subcutaneous or intracerebral dose	Kelly et al., (1968)
		Single intraperitoneal dose	Frei (1970, 1971a)
		Single or repeated intraperitoneal doses	Joshi and Frei (1970)
	Stomach	A few oral doses	Druckrey et al. (1961)
	Lung	Single subcutaneous or intracerebral dose	Kelly et al. (1968)
		Single intraperitoneal dose	Frei (1970, 1971a)
Hamster	Skin	Local applications	Graffi et al. (1967)
Rabbit	Brain & nerve tissue	Repeated intravenous doses	Jänisch and Schreiber (1967)
Guinea pig	Stomach and Pancreas	A few oral doses	Druckrey et al. (1968)

MMS is known to be a weak carcinogen and its tumour inducing ability is summarised in Table 2.

D. Historical aspects of lymphoma

Hodgkin's disease (not under this name) was the first neoplastic lesion of the haemopoietic system to be recognised in man; the morbid changes of the affected organs in this disease were first described by Hodgkin (1832). Cases of leukaemia were reported almost simultaneously by Craigie and Bennet (1845) and by Virchow (1846). Considering that the haematological changes were part of a definite pathological process involving certain organs of the body Virchow (1846) introduced the term leukaemia. He recognized two forms of leukaemia; in one, the small forms of white cells predominated and enlargement of lymph nodes was common; in the second, the large white cells increased in number and splenomegaly occurred. Myeloid leukaemia as a disease entity was established by Neumann (1870). Kundraat (1893) recognised lymphosarcoma (malignant lymphoma) as a disease entity different from leukaemia.

The earliest report of murine lymphoma was by Eberth (1878). Haaland (1905) gave the best of the early descriptions of the disease, noting the excessive enlargement of lymph nodes, a mediastinal tumour, and infiltration of liver, kidney and spleen.

E. Spontaneous lymphoma in mice

AKR and C58 are the two strains of mice that have an over 90% incidence of spontaneous lymphoma of the lymphoblastic type (Potter and Richter, 1932; Metcalf, 1966a). Potter et al. (1943) found that the in-

TABLE 2

Carcinogenicity of Methyl methanesulphonate

Species	Organ	Treatment	References
Rat	Brain & Nerve tissues	Single intraperitoneal and three intraperitoneal doses	Swann and Magee (1969)
Mouse	Lung, Liver, Stomach and Leukaemias	Continued oral doses	Clapp et al. (1968)

itial neoplastic changes involved the lymph nodes and the liver. Although the disease became widespread in the later stages, no mention of thymus involvement was made in the paper. It is now known that in AKR lymphoma the thymus is the site of origin and the peripheral blood may or may not show increased number of abnormal cells (Metcalf, 1966a). In mice of the SJL/J strain spontaneous lymphoma is of reticulum cell type and originates in the mesenteric lymph nodes and in Peyer's patches (Siegler and Rich, 1968). In mice of the CFW strain spontaneous lymphoma is extremely rare and exclusively thymic in origin (Frei, personal communication).

F. Induced lymphoma in mice

a) Radiation: The first successful induction of malignant lymphoma resulted from irradiation (Krebs et al., 1930). Kaplan (1967) has extensively studied radiation induced lymphoma; it was thymic in origin and involved also the lymph nodes, spleen, liver, and other parenchymatous organs.

b) Chemicals: Kirschbaum et al. (1940) and Walburg (1971) studied lymphomas induced by methylcholanthrene. Fiore-Donati et al. (1961) reported induction of lymphomas in new born mice by urethane. Rappaport and Baroni (1962) and Ball and McCarter (1968) were able to induce lymphomas by DMBA (7,12-dimethylbenz[a]anthracene). During experiments with skin papilloma production in ICR mice by painting anthralin (1,8,9-trihydroxyanthracene) in acetone, Yasuhira (1968) observed the development of other tumours including lymphomas. Further, the incidence was seen to increase by intraperitoneal injection. Lymphomas have also been shown to develop in adult and new born mice following treatment with methyl-

nitrosourea (Graffi and Hoffmann, 1966; Terracini and Stramignoni, 1967; Kelley et al., 1968; Frei, 1970; Joshi and Frei, 1970) and also after treatment with dimethylnitrosourea (Hiraki, 1971).

c) Viruses: Gross (1951) inoculated a group of new born C3H mice with a cell-free filtrate prepared from lymphomatous tissues of AKR mice with spontaneous or transplanted lymphoma. Within 24 to 35 weeks about 28% of the mice developed lymphoma while the incidence in the controls was very low (less than 0.5%). Later Gross (1957) developed a potent stable strain of the virus by repeated cell-free passage in susceptible mice permitting the confirmation of viral etiology of murine lymphoma. In the same year Friend (1957) reported the development of a biphasic disease following the inoculation of a Swiss strain with cell-free extracts of Ehrlich ascites carcinoma. The disease was characterized by splenic enlargement; the first phase was associated with diffuse proliferation of erythroid cells and the second phase was associated with diffuse proliferation of the reticulum cells. Rauscher (1962) isolated a potent virus from splenic tissues of BALB mice and the disease was also biphasic. In the first phase there was huge splenomegaly due to proliferation of erythroid cells and in the second phase spleen, lymph nodes and thymus were enlarged due to proliferation of lymphoid cells. There were several other reports of lymphoma induction in mice upon inoculation of cell-free extracts prepared from a transplantable mouse sarcoma (Moloney, 1959) and from an X-ray induced lymphoma of C57 black mice (Lieberman and Kaplan, 1959). A large number of reports on the induction of lymphomas by cell-free materials from a variety of sources have since appeared in the literature (review: Rich, 1968).

d) Hormones: Lymphomas induced by oestrogenic hormones have been described by Gardner et al. (1944). The thymus was the site of origin with dissemination to other lymphoid tissues. Law (1947) reported an increased incidence of spontaneous lymphoma in C58 mice by orchidectomy in mature males or by adrenalectomy in immature males and females. In parallel to the observation of Law (1947), the incidence of radiation-induced lymphoma was seen to be reduced by the administration of testosterone (Block, 1966).

e) Viruses in chemically and hormonally induced lymphomas: Several reports have appeared in the literature indicating that cell-free extracts prepared from chemically induced lymphomas in mice (Irino et al., 1963; Toth, 1963; Ribachi and Giraldo, 1966; Haran-Ghera, 1967; Ball and McCarter, 1971) and possibly even oestrogen induced lymphomas (Kunii et al., 1965) induce lymphomas in the recipients. This evidence may suggest that strains of mice with a low incidence of spontaneous lymphoma harbour a latent virus and that exposure to various physical and chemical agents activates the virus leading to the development of lymphoma.

f) Combination of agents inducing lymphomas: Kirschbaum et al. (1953) investigated the effects of three leukaemogens - X-rays, oestrogenic hormones and methylcholanthrene in BALB/c, CBA and DBA mice. In BALB/c and CBA mice, X-rays and oestrogenic hormones acted synergistically while methylcholanthrene was ineffective. On the other hand, in DBA mice oestrogenic hormones enhanced the oncogenic potency of methylcholanthrene and X-rays. Other workers (Toth et al., 1962; Nishizuka and Slusa, 1968) have also demonstrated the synergistic effects of com-

bination of lymphoma-inducing agents such as X-rays, methylcholanthrene, and DMBA and hormones.

G. DNA analysis

a) Isolation and purification: DNA occurs naturally in association with proteins and its separation from them and from other cellular constituents such as RNA and polysaccharide constitutes the problem of isolation. The problem is further influenced by the sensitivity of DNA to degradation by changes in pH, by physical damage and by nucleases present in the cell. During isolation of DNA, it is therefore essential to use reagents that inhibit or preferably put out of commission the nucleases and at the same time leave the native structure of DNA nearly intact.

The use of detergent for inhibiting of enzymes, breaking of the DNA-protein complex and removing of proteins was introduced by Marko and Butler (1951), and a similar method using sodium dodecyl sulphate was developed by Kay and his associates (1952). The detergent method, although widely used, is rather time consuming. The phenol method of Kirby (1957, 1959) is technically easier than the detergent method and is particularly suited for use with small samples of mammalian tissues. The yield of DNA and the amount of residual protein depend upon the salt used in association with phenol and empirically the best result is obtained by using a salt such as 4-aminosalicylate (Kirby, 1958). Addition of 8-hydroxyquinoline and m-cresol to the phenol results in an improved deproteinizing mixture (Kirby, 1962, 1965).

Other than protein, contamination with polysaccharides is occasionally a difficulty in the purification of DNA. They can be removed from DNA of most mammalian tissues by the methoxyethanol-phosphate

procedure (Kirby, 1956) or by ultracentrifugation (Kirby, 1964).

Separation of DNA from RNA may be achieved by incubating with ribonuclease and then precipitating the DNA with cold 2-ethoxyethanol (Kirby, 1967); alternatively, phenolphthalein diphosphate may be used in combination with phenol (Kirby, 1961).

b) Hydrolysis: So far as the hydrolysis of alkylated DNA is concerned no one method has been found to yield all the products in a suitable form. The reasons are that the alkylated nucleoside residues are in several instances unstable either to alkali (as 1-, 3-, 7-alkyladenosine; 3-alkylcytidine; 7-alkylguanosine - Lawley and Shah, 1972a) or to acid (O^6-alkylguanosines are dealkylated - Lawley and Thatcher, 1970) particularly with increasing temperature. It seems, therefore, preferable to isolate the products of alkylation in the form of bases.

Most generally, the liberation of bases from chemically modified DNA is achieved by some form of acid hydrolysis (Loring, 1955). The glycosidic bond in purine deoxyribonucleotides is very susceptible to acid hydrolysis, and in some instances the alkylated purines are liberated at an appreciable rate even at neutral pH (Lawley and Brookes, 1963).

The complete liberation of purines requires relatively stronger conditions than would suffice to hydrolyse isolated purine deoxyribonucleotides due to the insolubility of the initially produced polynucleotides in dilute acid. The methods that have been reported to be useful are: HCl, pH 1.6, 37°C for 24 hours or pH 2.8, 100°C for 1 hour (Tamm et al., 1952); HCl, pH 1.0, 37°C for 16 hours (Lawley and Thatcher, 1970); HCl, pH 1.0, 70°C for 30 minutes (Brookes and Lawley, 1971); HCl, pH 0.1, 100°C for 1 hour (Smith and Markham, 1950; Magee and Farber, 1962). Such

acid hydrolysis leaves the pyrimidine bases as oligonucleotides of the type $Py_n p_{n+1}$ as a consequence of the partial hydrolysis of apurinic acids by the B-elimination mechanism (Brown and Todd, 1955). Interest in the precise conditions used for acid hydrolysis has been stimulated following the demonstration that, as already stated, 0^6-alkylguanines are unstable to acid hydrolysis (Lawley and Thatcher, 1970).

In order to obtain pyrimidine bases, more vigorous hydrolytic conditions are necessary: 7.5 N perchloric acid, 100°C for 1 hour (Marshak and Vogel, 1950) 88% formic acid at 175°C for 30 minutes (Wyatt and Cohen, 1953). An alternative method for hydrolysis of alkylated pyrimidine nucleotides consists in refluxing with 50% HBr for 3 hours (Price et al., 1968).

c) Separation of the products: The most widely used method of separation of DNA hydrolysis products is chromatography on paper. This technique requires no special or expensive apparatus, it is reasonably rapid and can separate most non-volatile products which are then detectable by simple techniques. Disadvantages are that only comparatively small amounts of material can be applied to each paper (this may be an advantage when dealing with a very small amount of highly radioactive DNA), and in some cases different products have more or less similar Rf values even in many different solvent systems.

The separation of nucleic acid bases on paper requires the use of strongly acid or alkaline solvents, since only in such solvents will guanine be sufficiently soluble to migrate and its tendency to form 'tails' will be avoided (Wyatt, 1955; Brookes and Lawley, 1971). Up to the present date a large number of solvent systems have been described for sep-

aration of bases but relatively few of these show real advantages over others. Aqueous n-butanol, with and without added ammonia was one of the first solvent tested for this purpose (Vischer and Chargaff, 1948) and this still remains among the most useful. Other commonly used solvent systems are: Isopropanol - conc. HCl - water - 170:41:39 v/v, Methanol - conc. HCl - water - 7:21:1 v/v (Brookes and Lawley, 1971) and Isopropanol - conc. NH_4OH - water - 7:1:2 v/v (Lawley and Thatcher, 1970).

The greatest difficulty is encountered when separation is attempted of the constituents of a mixture containing normal as well as altered components as may be obtained from the hydrolysis of alkylated DNA. It is found with most of the solvent systems that either some pairs of alkylated bases are inadequately separated (Brookes and Lawley, 1971) or one or the other derivative runs very close to its normal counterpart making separation practically impossible. Even if they are separable theoretically in a particular solvent system, it may not be useful with the usual quantity of DNA hydrolysate that is spotted on paper. Since the overall alkylation is usually very low, the products will not be seen under UV light and their quantity cannot usually be measured by elution and spectrophotometry. The use of labelled agents to facilitate detection makes the situation more complicated. Not only do the products become labelled but also the normal bases due to metabolic incorporation (Brookes and Lawley, 1971). In a radiochromatogram developed in an apparently suitable solvent system, the separation zone between the two products, especially between a major and a minor one if they are quite close, may often be completely blurred due to overlapping radioactivity which is then revealed as a broad shoulder of the major "peak" in the graphic representation of the chromatogram. It is very difficult to solve this

problem in a single system and even two-dimensional chromatography may prove inadequate (Brookes and Lawley, 1971). Satisfactory resolution is, however, possible by re-chromatography in different systems (Heppel, 1967).

For ready reference, a long table showing the Rf values of purines, pyrimidines and their derivatives in nine different solvents has been compiled by Fink and Adams (1966).

Unidimensional multiple development technique which although proved very successful for the resolution of closely related compounds is an infrequently used procedure in paper chromatography. This technique was first proposed by Jeanes et al. (1951) and entails repeated irrigation of the chromatographic support with one or more solvents along the same direction. The theory of multiple chromatography, its practical and theoretical potentialities and the limitations were elaborately discussed by Thoma (1963a). The rationale behind the use of this technique is that very similar compounds which are partially separated on one solvent irrigation can, in most instances, be completely resolved by further solvent irrigations. On the other hand, there is never any guarantee that replacing one solvent by another will ever lead to complete or even increased resolution of closely related solutes.

A simple formula for the multiple development of amino acids for strip or circular chromatography may be found to be generally applicable (Chakrabortty and Burma, 1956). The rule states: the distance between two substances will increase in the second development if Rf_1 + Rf_2 is less than 1. There are a number of developments with the same solvent in the same direction which yield the greatest separation between two substances. Below or above this number, the extent of separ-

ation decreases. The equation describing this optimum of development is:

$$n_{opt} = \frac{\log \left(\frac{\log (1 - Rf_2)}{\log (1 - Rf_1)} \right)}{\log \left(\frac{(1 - Rf_1)}{(1 - Rf_2)} \right)}$$

where Rf_1 and Rf_2 are the Rf values of the two compounds after one development. Rf_1 is greater than Rf_2 (Rüdiger and Rüdiger, 1965).

Computer prepared tables relating the Rf values of two solutes to the number of solvent passes (p) required for their separation by a preselected distance have been compiled by Thoma (1963b).

III. EXPERIMENTAL WORK

Section A. Tumour induction by Methyl methanesulphonate (MMS)

1. Materials and Methods

Mice: Six to eight week old CFW/D Swiss mice (Ball, Huh and McCarter, 1964) of either sex, originally obtained from Carworth Farms, New City, New Jersey, and inbred by strict brother x sister mating for more than 50 generations were used. They were housed in plastic cages in groups of up to six to a cage, kept in air-conditioned quarters with an artificial light cycle, and fed Purina fox chow (The Ralston-Purina Company, St. Louis, Mo.) and tap water *ad libitum*.

Carcinogen treatment: MMS (obtained from K & K Laboratories, Plainview, N.Y.) was used as purchased. It was dissolved in cold Standard Saline Citrate (SSC: 0.15 M Sodium Chloride, 0.015 M Sodium citrate) immediately before use and injected intraperitoneally within one hour after dissolution.

In previous experiments in this laboratory, the LD_{50} dose of MMS in this strain of mice was found to be approximately 176 mg/Kg body weight (1.6 mmoles/Kg). A dose below the LD_{50}, 110 mg/Kg (1.0 mmoles/Kg) was chosen for these experiments. Two schedules were used: one group of 27 female and 3 male mice was injected with a single dose and another group of 30 mice with the same sex distribution was injected weekly with the same dose for 5 weeks. Since repeated controls were done recently in the laboratory with this strain of mice with uniformly similar results, no untreated control group was done in these experiments.

<u>Parameters studied</u>: The mice were observed for early mortality, i.e., death within 3 weeks of the last injection of MMS, and for the incidence of tumours. The experiments were terminated at 258 days after the first injection.

Those mice that died before termination, or were sacrificed at the end of the observation period, were carefully autopsied and all tumour-like lesions were examined histologically. Only microscopically confirmed tumours were used for final accounting.

Tumour yield was compared by a chi-square test using Yates correction (Quenoville, 1969 ed.).

2. Results

The results on the early mortality and tumour incidence are shown in Table 3, which also includes recent control and MNUA data from this laboratory.

There was no early mortality. Two mice, one from each group died before termination (at 187 days in the single dose group and at 230 days in the fractionated dose group) and autopsy revealed no apparent abnormalities.

There was no significant difference in tumour yield between untreated controls and either group of MMS-treated mice.

TABLE 3

TUMOUR INDUCTION BY METHYL METHANESULPHONATE AND METHYNITROSOUREA IN SWISS MICE

Group No.	Agent	mM/Kg Approx. LD_{50}	mM/Kg Dose used	Duration of Expt. (days)	Number of early deaths	Effective no. of mice[a]	Lymphomas Number	Lymphomas %	Lung adenomas Total No.	Lung adenomas No./mouse
1[b]	-	-	-	210	0	29	0	0	4	0.14
2[b]	-	-	-	365	0	103	3	3	3	0.003
3[b]	MNUA	1.4	1.2	365	14	36	26	72	25	0.70
4[c]	MNUA	1.4	0.5	250	0	24	14	58		
5[c]	MNUA	1.4	0.75	250	2	35	25	72		
6[c]	MNUA	1.4	0.4 x 5 (weekly)	250	0	29	27	93		
7	MMS	1.6	1.0	258	0	30	0	0	1*	0.03*
8	MMS	1.6	1.0 x 5 (weekly)	258	0	30	0	0	1*	0.03*

a Mice living longer than 3 weeks after the last injection

b Data from Frei (1971a)

c Data from Joshi and Frei (1970)

* Statistically not significantly different from controls (Groups 1 & 2) at the 1% level.

Section B. Tissue changes following treatment with Methyl methanesulphonate (MMS), Ethyl methanesulphonate (EMS), Ethylnitrosourea (ENUA) and Methylnitronitrosoguanidine (MNNG)

1. Materials and Methods

Mice: Adult 6 to 8 weeks old CFW/D female Swiss mice were used. They were kept under the conditions as described in Section A.

Carcinogen treatment: Four compounds were tested: MMS (K & K Laboratories, Plainview, N.Y.), EMS (Eastman Organic Chemicals, Rochester, N.Y.), ENUA (K & K Laboratories, Plainview, N.Y.), and MNNG (K & K Laboratories, Plainview, N.Y.) All these compounds were used without further purification with the exception of ENUA which was recrystallized from ethanol before use. MMS, EMS and ENUA were injected dissolved in SSC while MNNG was injected dissolved in 0.9% NaCl by warming up to about 50°C and vigorous shaking. Because of the known photosensitivity of the nitroso compounds, solutions of ENUA and MNNG were made and used in subdued light. All injections were given within one hour of dissolution of the drugs.

The toxicity of the four compounds had been determined previously and the same dose for each used in previous tumour induction experiments (Frei, 1971a; Frei and Joshi, 1973) was used for this study. The dose in mmoles/Kg body weight was 0.4 for MNNG, 1.2, for MMS, 2.0 for ENUA and 3.0 for EMS.

Groups of 6 mice were given a single dose of one of these drugs intraperitoneally in a maximum volume of 0.5 ml.

The control group consisting of 12 mice was given nothing.

Parameters studied: For each drug, 6 groups of mice were examined for tissue changes at various intervals - one group each at 1, 3, 6, 10, 15 and 20 days after the injection. The mice were killed by ether and the gross appearance of all the organs noted. The fresh weight of the spleen and of the thymus dissected as free of fat as possible was determined; the bone marrow was flushed out from a standard full length of one femur with SSC and the number of nucleated cells counted in a haemocytometer. The mice belonging to the control group were similarly examined at 0 day.

Data at each point, consisting of 6 results for each tissue were analysed statistically by comparison of the means with the control (0 day) group and the results giving a p-value of <0.01 were considered significant.

2. Results

The results are shown in Table 4 and in Figures 1 to 3.

It can be seen that the one compound which was found to induce thymic lymphomas, ENUA (Frei, 1971a), caused the maximum weight loss or a cell depletion in all the three tissues. It can also be seen that MMS, and the two other compounds EMS and MNNG, which were not found previously to induce thymic lymphomas, did not cause a significant depression of cell count in the bone marrow although significant reductions in weight of the thymus were produced by MMS and EMS and of spleen by EMS. This loss in weights of spleen and of thymus was reflected in the gross appearance of

the organs, i.e., during dissection the organs appeared smaller and shrunken. In other organs particularly the liver and the kidneys no apparent gross changes were seen.

It can also be noted that the recovery of the bone marrow was fastest and that of thymus was slowest; in any case, the recovery was complete by about 10 days.

TABLE 4

TISSUE CHANGES FOLLOWING ADMINISTRATION

OF MMS, EMS, ENUA AND MNNG

IN SWISS MICE

TISSUE	Control	MMS GROUP					
	0 day	1 d	3 d	6 d	10 d	15 d	20 d
Bone marrow x 10^6 cells	7.23	6.07	7.23	9.23	8.96	7.1	10.7
Spleen wt in mgm	81.1	59.7	93.5	88.5	95.6	93.1	94.0
Thymus wt in mgm	40.2	25.7	21.8*	29.7	43.7	40.1	37.2

TISSUE	Control	EMS GROUP					
	0 day	1 d	3 d	6 d	10 d	15 d	20 d
Bone marrow x 10^6 cells	7.23	4.97	5.63	7.93	8.0	9.05	8.47
Spleen wt in mgm	81.1	42.0*	48.4	65.0	95.2	84.9	78.2
Thymus wt in mgm	40.2	23.7*	15.4*	22.9*	56.3	49.7	42.8

* $p<0.01$ in comparison with control values

Contd....

TABLE 4

TISSUE CHANGES FOLLOWING ADMINISTRATION

OF MMS, EMS, ENUA AND MNNG

IN SWISS MICE

TISSUE	Control	ENUA GROUP					
	0 day	1 d	3 d	6 d	10 d	15 d	20 d
Bone marrow x 10^6 cells	7.23	1.5*	2.73*	5.66	7.77	8.13	9.5
Spleen wt in mgm	81.1	43.8*	35.6*	52.0	118.1	93.5	94.2
Thymus wt in mgm	40.2	23.6*	15.5*	24.4*	45.2	40.3	41.2

TISSUE	Control	MNNG GROUP					
	0 day	1 d	3 d	6 d	10 d	15 d	20 d
Bone marrow x 10^6 cells	7.23	5.13	8.16	9.63	11.13	7.87	10.63
Spleen wt in mgm	81.1	56.5	65.9	98.9	92.3	107.6	105.9
Thymus wt in mgm	40.2	49.1	31.6	26.8	44.2	35.5	40.4

* $p<0.01$ in comparison with control values

Fig. 1. Counts of bone marrow cells from single femurs of adult female inbred Swiss mice given a single sub-lethal intraperitoneal dose of 1.2 mmoles MMS/Kg., 3.0 mmoles EMS/Kg., 0.4 mmoles MNNG/Kg., or 2.0 mmoles ENUA/Kg. body weight at various intervals. Each symbol represents the mean measurement made on 6 mice. Empty symbols represent data where the mean value is significantly different from control value at the 1% level.

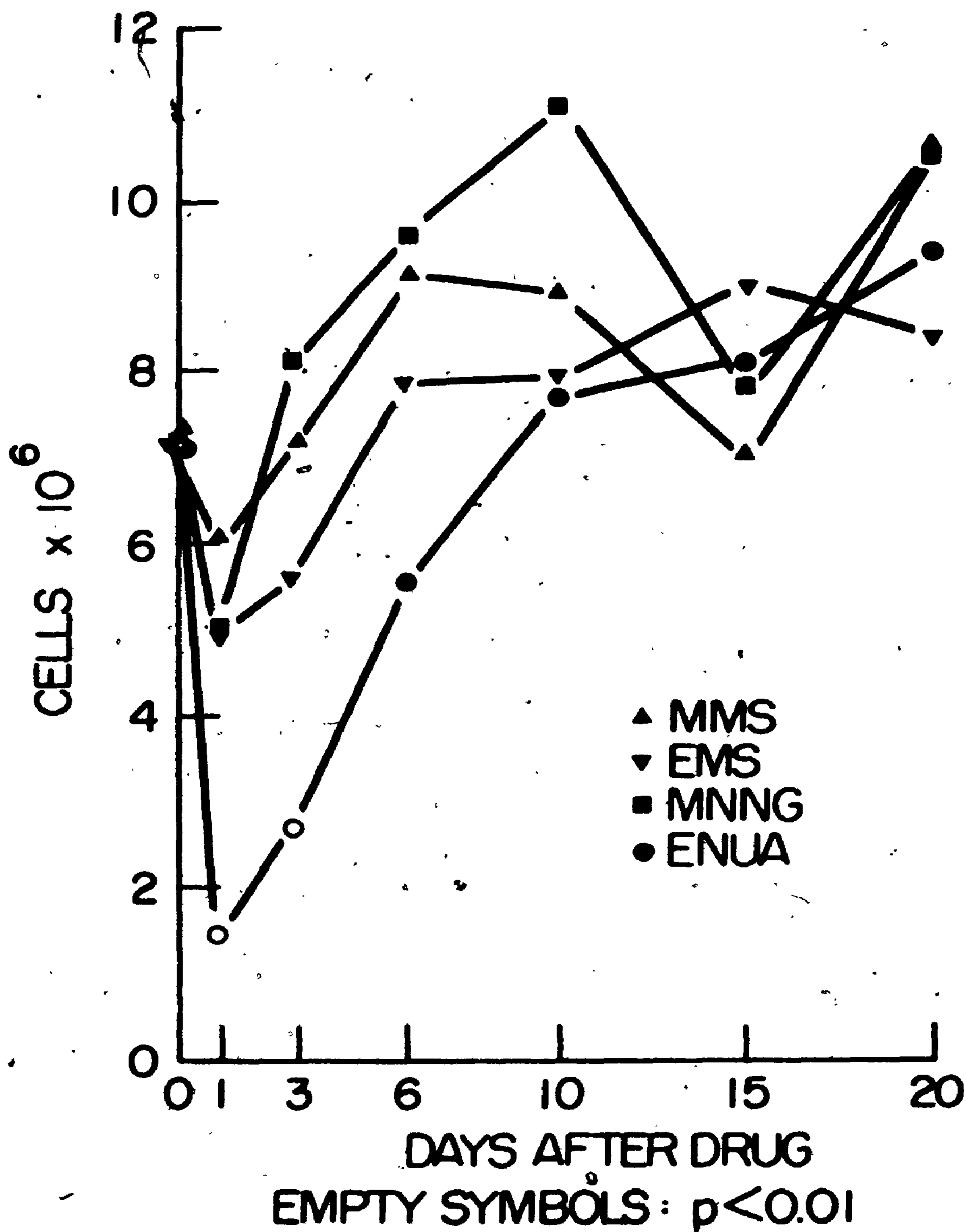


FIG. 1

Fig. 2. Thymus weights of adult female inbred Swiss mice given a single sub-lethal intraperitoneal dose of 1.2 mmoles MMS/Kg., 3.0 mmoles EMS/Kg., 0.4 mmoles MNNG/Kg., or 2.0 mmoles ENUA/Kg. body weight at various intervals. Each symbol represents the mean measurement made on 6 mice. Empty symbols represent data where the mean value is significantly different from control value at the 1% level.

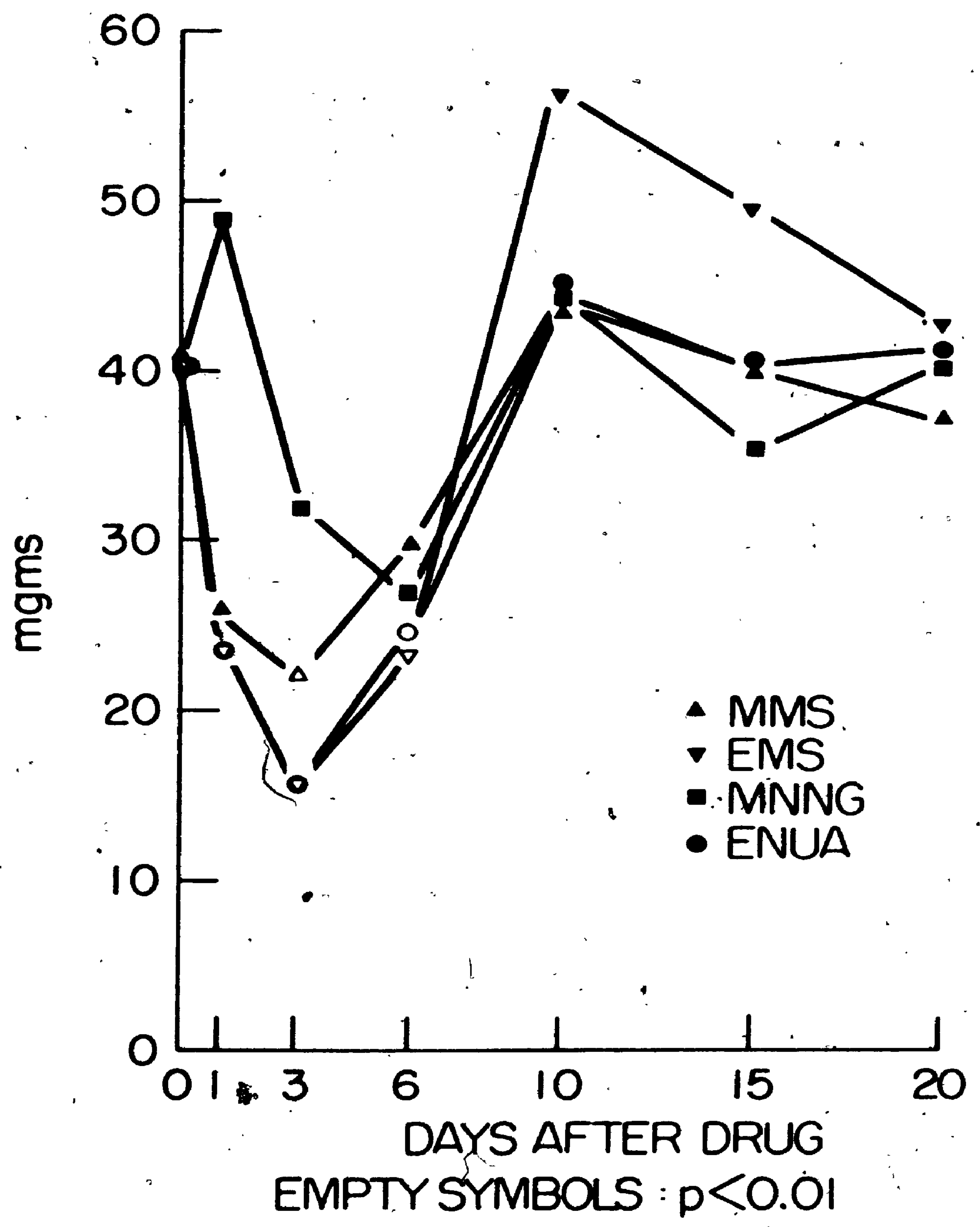


FIG. 2

Fig. 3. Spleen weights of adult female inbred Swiss mice given a single sub-lethal intraperitoneal dose of 1.2 mmoles MMS/Kg., 3.0 mmoles EMS/Kg., 0.4 mmoles MNNG/Kg., or 2.0 mmoles ENUA/Kg. body weight at various intervals. Each symbol represents the mean measurement made on 6 mice. Empty symbols represent data where the mean value is significantly different from control value at the 1% level.

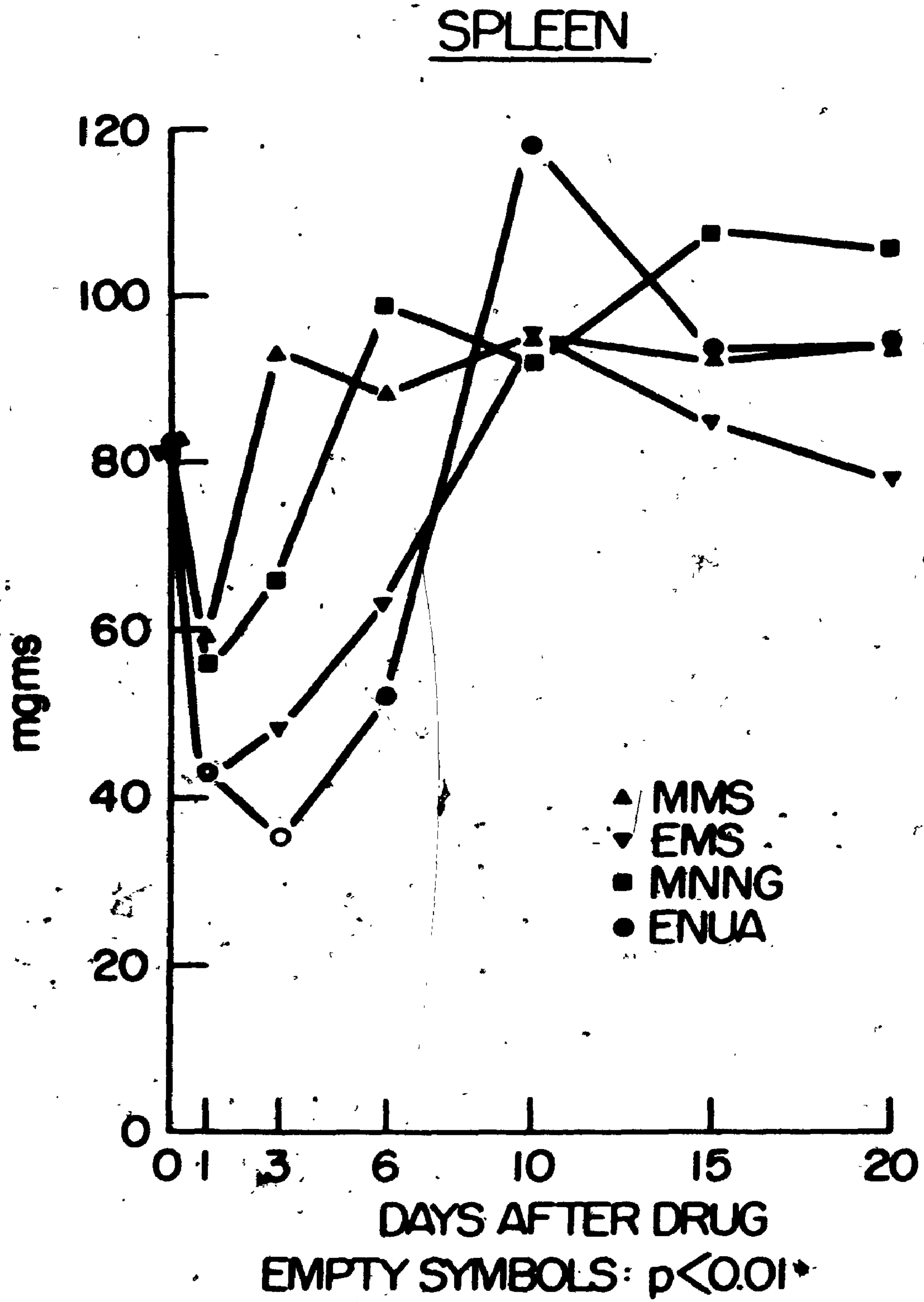


FIG.3

Section C. Reaction products of DNA following treatment with ^{14}C-methyl-nitrosourea (^{14}C-MNUA) and ^{14}C-methyl-methanesulphonate (^{14}C-MMS)

1. Materials and Methods

Mice: Adult 6 to 8 week old CFW/D female Swiss mice kept under the same conditions as described in Section A were used throughout these experiments.

Radioactive compounds: Several batches of ^{14}C-methyl-nitrosourea were prepared from ^{14}C-methylurea (Sp.activity 5.94 mCi/mmole) obtained from NEN Canada Ltd. (Dorval, Quebec, Canada) by nitrosylation (Murray and Williams, 1958). It was stored desiccated at about -5°C until used. The final specific activities of the batches of ^{14}C-methyl-MNUA were calculated from their ultraviolet absorption spectra compared to recrystallized non-radioactive MNUA spectrum (in a Cary 15 spectrophotometer) and from their radioactivities determined by scintillation counting (in a Packard Tri-carb liquid scintillation counter - Model 3375) using toluene internal standards. The specific activities as determined for different batches were 1.65, 1.83, and 2.08 mCi/mmole.

^{14}C-methyl-methanesulphonate (Sp.activity 56 mCi/mmole) in ether solution was obtained from Amersham/Searle Company (Don Mills, Ontario, Canada). It was stored at about -5°C until used. As it was used suitably diluted with non-radioactive MMS in different experiments, the final specific activities were calculated from the dilution factors (i.e., 1.36, 1.41, 1.74, and 1.75 mCi/mmole).

<u>Carcinogen treatment</u>: Just before use, the ethereal solution of ^{14}C-methyl-MMS was diluted with non-radioactive MMS (K & K Laboratories, Plainview, N.Y.) according to the need and the ether quickly blown off by a gentle stream of cool air.

Both ^{14}C-MMS and ^{14}C-MNUA were dissolved in cold SSC immediately prior to treatment and injected within one hour of the dissolution of the drugs. Because of the known photosensitivity, the solution of ^{14}C-MNUA was made and used in subdued light.

The same dose for each compound used in previous experiments was used for this study. The dose in mmoles/Kg body weight was 1.2 for ^{14}C-MMS and 0.6 for ^{14}C-MNUA.

Groups of 3 or 4 mice were given a single dose of one of these drugs intraperitoneally in a maximum volume of 0.5 ml.

<u>Parameters studied</u>: For each drug, 5 groups of mice were examined for the methylation products of DNA obtained from various tissues like gut, liver, kidney, lung, spleen, thymus and bone marrow, at different intervals by independent experiments - one group at each of 0.3, 1.0, 2.0, 4.5, and 12 hours after the injection.

Further, the gross appearance of the organs during dissection and the overall yield of DNA from different tissues were noted.

<u>Procedure for isolation and purification of DNA</u>: The reagents used were:

a) Aq. 6% (w/v) sodium-4-aminosalicylate (British Drug Houses Ltd., England)

b) Aq. 10% (w/v) sodium dodecyl sulphate (Fisher Scientific Co, USA)

c) Phenol reagent (Kirby, 1967): Liquid phenol 946 ml; m-cresol 140 ml; 8-hydroxyquinoline 1.0 g. (all from Fisher Scientific Co, USA)

d) Aq. 5% (w/v) sodium acetate (British Drug Houses Ltd., England)

e) 2-ethoxyethanol (Fisher Scientific Co. USA)

f) Ethanol (Canadian Laboratory Supplies Ltd., Canada)

g) Di-ethyl ether (Mallinckrodt Chemical Works, USA)

h) Ribonuclease - dry (Worthington Biochemical Corporation, N.J., USA)

In all the experiments, the method employed for DNA isolation and purification was basically that of Kirby (1957) with modifications as described by Lawley and Thatcher (1970).

At specified time, the animals were killed one at a time by ether anaesthesia and DNA isolated from pooled organs. Some time spread could not be avoided due to dissection and in no case was it more than 20 minutes. In short time-course experiments such as at 0.3 hours, the injections were given to animals adequately spaced (10 minutes) to allow time for dissection.

Thymuses, Livers, Gut (both large and small - lumen flushed out with buffered phosphate saline), Kidneys, Lungs, Spleens and Bone marrow flushed out from tibiae and femora of animals were plunged into ice-cooled tubes containing 6% PAS - 15 ml for each pool of liver and gut and 10 ml for each of the other organ pools. The tissues were homogenized in a teflon-glass homogenizer in the cold solution, 0.1 volume of 10% SDS was added and the viscous lysate was shaken manually for about

10 minutes with an equal volume of phenol reagent. The mixture was then centrifuged at 17000 x g for 30 minutes at 5°C. The upper aqueous layer was removed by pipetting and fibrous DNA precipitated by slow addition of 1.5 volume of 2-ethoxyethanol and gentle mixing. The precipitated DNA was allowed to stick to a glass rod by mild swirling movement of the rod and the ethoxyethanol was drained off. The DNA was immediately re-dissolved in the original volume of 5% sodium acetate and incubated with a small amount of ribonuclease at 37°C for 1 hour. A second extraction was done with phenol reagent and the aqueous layer clarified by centrifugation in an MSE 65 ultracentrifuge at 80000 x g for 1 hour at 20°C. The DNA fibre was then obtained by precipitation with 2 volumes of cold ethanol, it was washed 3 times with ethanol and 3 times with ether, dried in air and stored at 5°C up to the time of hydrolysis.

Hydrolysis: The method of hydrolysis was based on that of Lawley and Thatcher (1970) and that used previously in this laboratory (Frei, 1971b). Because there was known to be little alkylation of pyrimidines (Lawley and Thatcher, 1970), the main purpose was to release all of the alkylated purines with as little destruction as possible. Some form of mild acid hydrolysis, capable of releasing all purines and alkylated purines (Loring, 1955) appeared potentially suitable judging from previous work (Frei, 1971b; Lawley, 1972). The reported procedures using 0.1 N HCl were tried, but none could produce the desired and consistent degree of degradation as judged by subsequent radio-chromatographic fractionation. 0.1 N HCl was then tried under various conditions of temperature and time. The stability of some of the known methylated purine was

tested under such conditions using authentic compounds 7-methylguanine, 3-methyladenine, 3-methylguanine, 1-methyladenine, and 0^6-methylguanine which were obtained either commercially (Cyclo Chemical, Los Angeles, California, USA, and Sigma Chemical Co., St. Louis, USA) or courtesy of Dr. P. D. Lawley of Chester Beatty Research Institute, England. The eventually adopted procedure of hydrolysis was heating with 0.1 N HCl at $100^{o}C$ for 30 minutes.

In order to determine the stability of purine methylation products, 1 mg of each of the five authentic and other related compounds was dissolved in 1 ml of 0.1 N HCl and diluted either 1: 100 or 1: 200 with 0.1 N HCl. Ultraviolet absorption spectra of the solutions were determined in a Cary 15 spectrophotometer before and after heating at $100^{o}C$ for 30 minutes. There was no change in the spectra except with 0^6-methylguanine. Since this product is known to degrade to guanine by acid hydrolysis (Lawley and Thatcher, 1970), the extent of degradation, expressed in terms of molar percentage, was estimated by comparing the spectra of the mixture obtained by heating 0^6-methylguanine at $100^{o}C$ for 30 minutes and those of pure guanine and pure 0^6-methylguanine in 0.1 N HCl by the principle as suggest by Loring (1955).

"The analysis of a binary mixture of components, A and B, having different absorption spectra, is made on the assumption that the total optical density, D, at each of two wavelengths, λ_1, and λ_2, is the sum of densitites due to each component at each wavelength. Thus from the Beer-Lambert law two simultaneous equations can be written in which the concentrations of A and B are expressed as a function of their molecular extinction coefficients and the optical densities at the respective wavelengths as follows:

$$C_A E_A \lambda_1 + C_B E_B \lambda_1 = D\lambda_1$$

and

$$C_A E_A \lambda_2 + D_B E_B \lambda_2 = D\lambda_2$$

C_A and C_B are the respective concentrations of A and B expressed as moles per liter; $E_A\lambda_1$, $E_B\lambda_1$, $E_A\lambda_2$, and $E_B\lambda_2$ are the respective molecular coefficients of A and B at λ_1 and λ_2; and $D\lambda_1$ and $D\lambda_2$ are optical densities at the two wavelengths. Solution of the equations for C_A and C give the following:

$$C_A = \frac{E_B\lambda_1\ D\lambda_2 - E_B\lambda_2\ D\lambda_1}{E_A\lambda_2\ E_B\lambda_1 - E_A\lambda_1\ E_B\lambda_2} \quad \text{(i)}$$

$$C_B = \frac{E_A\lambda_2\ D\lambda_1 - E_A\lambda_1\ D\lambda_2}{E_A\lambda_2\ E_B\lambda_1 - E_A\lambda_1\ E_B\lambda_2} \quad \text{(ii)}$$

The degree of degradation under these conditions (see Results) was considered acceptable.

Because of the short period of time used for hydrolysis, it was found that consistent results were obtained only if two conditions were fulfilled: (i) the DNA fibre was dissolved in distilled water prior to the addition of acid and (ii) the dry weight of DNA was kept to 1 mg or less for 0.3 ml of 0.1 N HCl.

Batches of up to 1 mg of DNA were allowed to dissolve in 0.15 ml distilled water in screw-cap test tubes with intermittent shaking at room temperature until no visible undissolved material was noted. This took approximately 1 hour in all instances. 0.15 ml of 0.2 N HCl was then added to each tube and the solution mixed gently until a uniform

chalky-white suspension was formed. The tubes were then securely capped, heated in a water bath at 100°C for 30 minutes, and immediately cooled in ice-water. In each case a clear solution was obtained.

Chromatography: A form of unidimensional two-stage descending paper chromatography was devised for the separation of methylated purines. In all cases, the zone corresponding to O^6-methylguanine in the original chromatogram was re-chromatographed in a different solvent. In this study attention was focussed on the three known major methylation products, i.e., 7-methylguanine, 3-methyladenine and O^6-methylguanine and this governed the development of the present system of separation.

In developing this system, various solvents noted in the literature (Lawley and Thatcher, 1970; Fink and Adams, 1966) were first tested by standard descending chromatographic procedures. In each case, control runs with unlabelled markers were first carried out on 4 cm wide Whatman 3MM paper (45 cm run) at a room temperature of about 22°C - 25°C and whenever a particular solvent looked promising, it was extended to radiochromatography. None of the solvents tested proved completely satisfactory for the defined purpose. With quite a few solvents adenine, 3-methyladenine and O^6-methylguanine could be separated reasonably well, but the separation of guanine from 7-methylguanine was inadequate. The solvent containing t-butanol - methyl ethyl ketone - water - conc. ammonia - 40: 30: 20: 10 v/v (Fink and Adams, 1966) appeared somewhat better because in a run of 45 cm it not only separated adenine, 3-methyladenine and O^6-methylguanine but also showed some splitting of the confluent zone

of guanine and 7-methylguanine. To improve the situation, runs on paper previously washed with the solvent and dried, or on cellulose-phosphate paper (P 81) were tried but neither of these modifications proved to be of any real value. The usual alternate approach to the solution of this type of problem is to increase the effective distance of solvent flow by overrunning the end but since this technique might lead to some loss of radioactivity from the paper it was avoided. On the other hand, the length of the paper was actually increased; within the practicability of a standard size chromatography tank it was possible to make the effective length of run to about 59.5 cm. Even this procedure failed to show any significant improvement.

At this point, separations were tried with modifications of this solvent, i.e., increasing the proportion of ammonia and decreasing the proportion of water. By trial runs with varying proportions of ammonia and water, guanine and 7-methylguanine were found to be separated by up to 1.5 cm simultaneously with a wide separation of adenine, 3-methyladenine and O^6-methylguanine (over 4 cm in each case) in an effective run of 59.5 cm at the solvent composition of t-butanol - methyl ethyl ketone - water - conc. ammonia - 40: 30: 10: 20 v/v. Although this modified solvent appeared very promising, it was felt desirable to increase the distance between guanine and 7-methylguanine by at least another 1.0 cm because it was feared that on occasions this narrow zone of separation might get blurred when extended to radiochromatography and also there might be tailing of guanine.

For this purpose, it appeared worthwhile to try two-stage development which is essentially a multiple development technique. However,

the advantages of this multiple development technique could not be fully utilized in the present situation since the starting material was not a simple binary mixture but was a DNA hydrolysate containing a number of products. Except for guanine and 7-methylguanine, all other products which we were interested with, showed Rf values greater than 0.5 on first development and according to the rule (Chakrabortty and Burma, 1956), the distance between them would decrease with repeated developments. In fact, there was complete freezing of the fast migrating products, i.e., 3-methyladenine and O^6-methylguanine even on second development. All the available information concerning the usage of this technique considered the number of solvent passes (p) as an integer and in the present situation, this system turned out unsuitable even when p was 2.

The prospect of a modified form of multiple development technique using solvent passes more than 1 but less than 2 has not yet been looked into, but hopefully such a system should carry the same potentialities as others and since the present need was to increase the distance between guanine and 7-methylguanine by only about 1.0 cm without seriously affecting the position of other products, it was decided to exploit the modified two-stage technique, i.e., short first run followed by full second run in the same solvent. To determine the feasibility of the desired separation and the optimal length of first run needed for it, trials were conducted with different lengths of first run, i.e., 10, 15, 20, and 25 cm. Satisfactory results were obtained by a two-stage development - 20 cm first run followed by 59.5 cm second run. The reproducibility of this technique and the influence of other variables, e.g., temperature and the maturity of the solvent were then checked by repeating the procedure a

number of times. It was shown to produce ideal results if (i) development was carried out at a room temperature of 22° - 25°C, (ii) fresh solvent was used each time, and (iii) the paper strip was completely dried preferably over a hot plate at 60°C after the first development.

* * *

Because of the possibility that some pyrimidine bases might be liberated during the hydrolysis procedure and might be labelled with ^{14}C due to metabolic incorporation, hydrolysis and chromatography of ^{14}C-thymidine labelled DNA was performed. Two female mice were injected intraperitoneally with 0.5 ml (10 mCi) of thymidine-2- ^{14}C twice, at an interval of one hour and sacrificed one hour after the second injection. DNA was isolated from organ pools as before and hydrolyzed and chromatographed as indicated above. Authentic markers of thymine and 0^{6}-methylguanine were added to the chromatograms. It was found that 1.4 percent of the thymidine radioactivity was present at thymine location. It became necessary, as noted above, to re-chromatograph the zone corresponding to 0^{6}-methylguanine (marked always with the authentic compound) to separate it from thymine and from 3MeC which also travelled to a similar location (see Results).

As separation was desired between three compounds only, it was decided to save time by using a solvent which on short runs would be capable of separating them adequately. For this purpose, the solvent chosen was: upper phase of Ethyl acetate - formic acid - water - 60: 5: 35 v/v (Fink and Adams, 1966), and in runs of 29 - 30 cm effective distance a satisfactory separation of 3-methylcytosine, thymine and 0^{6}-methylguanine

was achieved.

* * *

Detailed procedure of the modified two-stage chromatography: To one end of a 4 cm wide Whatman 3MM paper of standard length (about 57 cm) another 15 cm long strip of the same width was sewn up one edge over the other with ordinary sewing thread. After allowing sufficient margin for covering the distance between the support and the antisiphon rods, three lines were drawn to indicate origin, the distance for the first run (20 cm from the origin), and the distance for the second run (59.5 cm from the origin). Keeping a further 0.5 cm of the strip, the redundant portion was cut off.

Following hydrolysis of DNA fibre, 2 x 20 µl aliquots were pipetted out, one for determination of total radioactivity and the other for UV spectrum recording; the remainder was then streaked on the paper in 50 µl lots together with some 0^6-methylguanine to act as a marker. The total volume of the hydrolysate streaked was noted. The chromatography tank was first allowed to saturate with the solvent vapour at room temperature, i.e., 22° - 25^oC, the solvent being placed in the trough and in beakers at the bottom. The chromatogram was then run at the same temperature for a distance of 20 cm (9 - 10 hours run), dried in air and then over a thermostatically controlled hot plate at 60^oC for about 10 minutes, and a second run was made exactly to 59.5 cm (25 - 26 hours run). In between runs, the used solvent in the trough and in the beakers were replaced by fresh solvent and the vapour inside the tank was re-equlibrated with that of the fresh solvent. After the run, the strip was dried in air, examined under UV light (254 mµ) and the position of 0^6-methylguanine as detected by

its brilliant blue fluorescence was marked with a pencil. The zone corresponding to 0^6-methylguanine (usually 39 - 42 cm) was then cut off in one piece for re-chromatography.

Detailed procedure for re-chromatography: In view of the possibility of loss of radioactivity during re-chromatography if elution from the first paper was employed, this procedure was avoided.

The same Whatman 3MM paper - 4 cm wide with no extra piece added was used for this purpose. As before, two lines were drawn to indicate origin and the distance for the run (25 cm from the origin). The strip was then cut along the origin line and the saved piece from the previous chromatogram was sewn upside down (in the reversed order) to the cut edges of the strip and chromatographed in the solvent as already described. The rationale behind sewing up the strip in the upside down fashion was to improve the separation of the products by taking advantage of their relative positions in the two solvent systems (see below). When the solvent moved up to the 25 cm line (3 - 4 hours run), the strip was taken out, dried in air and examined under UV light for the position of 0^6-methylguanine.

<u>Determination of degree of base alkylation</u>: Detection, identification and quantitation of the methylated purines were done by radioactive assay of the chromatogram assuming that the specific activity of the (^{14}C)-methyl group as was present in the drug remained unchanged after its reaction with DNA.

All radioactivity measurements were done in a Packard Tri-carb liquid scintillation counter - Model 3375. Each sample was counted 2 x 10

minutes and the average of the two counts was taken to be the measure of radioactivity of the particular sample. At each tissue level, total radioactivity was determined with 20 ul hydrolysate aliquot in 10 ml of a phosphor containing naphthalene (100 gm), 2,5-diphenyloxazole (4 gm), p-bis-2-5-phenyloxazolylbenzene (100.gm), methanol (100 ml) and dioxane (to 1 liter). Efficiency of counting liquid samples was about 75% for ^{14}C as determined with toluene internal standardization of the original hydrolysate aliquot. The efficiency was further checked by determining the linearity of counts with different dilutions of ^{14}C-toluene internal standard in 10 ml of the same phosphor. With the efficiency known, corrections from counts/min. to disintegration/min. were then made.

With paper chromatograms, sequential 1 x 4 cm segments were cut, placed individually in scintillation vials containing 0.5 ml of a phosphor containing 2,5-diphenyloxazole (4 gm), p-bis-2-5-phenyloxazolylbenzene (50 mg) and p-toluene (1 liter) and counted. When calculated, counting efficiency on paper was found to be about 50%. Total background-free radioactivity of the chromatogram was then graphically reproduced in which the products were shown up as 'peaks'. Such graphically delimited 'peaks' were taken to be the measure of the products and their identities established by comparing the migration values of reference compounds from chromatograms run in parallel. In a few cases initially, the identities were further checked by re-chromatographing the respective 'peaks' separately in suitable solvents along with markers.

For the purpose of attaching true significance to the 'peaks' in the radiochromatogram, particularly the smaller ones, it was felt necessary to standardize the background radioactivity. A full length

blank strip was chromatographed and counted exactly the same way as others; the standard deviation from the average of all the counts was calculated to be ± 1.01 counts/min. Assuming that a product would occupy at least 3 cm wide area in the chromatogram and that 99% of the determinations would lie within 2.58 s.d., any peak showing over 8.0 counts/min. was considered to be a true 'peak'.

The measure of total base methylation or of methylation of individual bases with respect to the different products were obtained respectively as: (i) total base methylation was calculated from the total radioactivity recovered on the chromatogram less radioactivity due to unidentified 'peaks' and due to ^{14}C incorporation into normal bases; (ii) individual base methylation of any one type was calculated from the radioactivity obtained under the appropriate individual 'peaks'. To quantitate, the specific activity of the DNA sample was calculated from the concentration of DNA as determined with UV absorption of 20 ul hydrolysate aliquot diluted with 3 ml distilled water; it was assumed that a solution containing 1 mg DNA/ml had a value E_{260} = 26 (Lawley and Thatcher, 1970) and that 1 mg of mouse DNA contained 0.00306 mM of bases (all four bases in molar proportion as under the native state) (Kit, 1960). From these data molar percentages of total base methylation and methylation of various types of individual bases were calculated.

It may be pointed out that in the chromatography system employed in the present study, 3-methylguanine has a migration value very close to that of 7-methylguanine (see below), and it is possible that the small amount of radioactivity due to 3-methylguanine could be completely submerged in that due to 7-methylguanine. This would not significantly

affect the quantitation of 7-methylguanine, because 3-methylguanine was reported to comprise only about 1% of the methylation products of DNA (Lawley, 1972).

2. Results

Degradation of 0^6-methylguanine: Using the principle suggested by Loring (1955), the data obtained when authentic 0^6-methylguanine was hydrolysed at 100°C in 0.1 N HCl for 30 minutes and the spectrum after hydrolysis compared to that of guanine and 0^6-methylguanine was as follows:

0^6-methylguanine	: at 270 mμ	E = 8.76 (x 10^{-3})
	at 250 mμ	E = 2.89 (x 10^{-3})
Guanine	: at 270 mμ	E = 10.90 (x 10^{-3})
	at 250 mμ	E = 16.85 (x 10^{-3})
The treated 0^6-methylguanine	: at 270 mμ	O.D. = 0.600
	at 250 mμ	O.D. = 0.387

Using equations (i) and (ii) (see above) it was calculated that the mixture obtained as above contained, in terms of moles, 78% 0^6-methylguanine and 22% guanine; therefore, the loss of 0^6-methylguanine was 22%. Lawley and Thatcher (1970) indicated that under the same conditions the half life of 0^6-methylguanine was 1.4 hours and from their data the loss at 30 minutes should be identical, i.e., 22 percent.

Location of product peaks: In the two-stage chromatography system it is not possible to obtain Rf values. The final location of the various products as determined using authentic compounds can be referred to as the migration value, that is the distance from origin to the center of the final spot. These were found to be:

Thymidine-5'-phosphate	:	11.5 cm
Guanine	:	18.0 cm
3-methylguanine	:	20.0 cm
7-methylguanine	:	21.5 cm
Adenine	:	30.0 cm
1-methyladenine	:	33.0 cm
Cytosine	:	34.4 cm
3-methyladenine	:	36.7 cm
Thymine	:	39.8 cm
3-methylcytosine	:	40.2 cm
0^6-methylguanine	:	41.0 cm

The re-chromatography of the 0^6-methylguanine region of the two-stage chromatogram in a second solvent by the method described above gave the following migration values for the three products concerned:

3-methylcytosine	:	1.0 cm
0^6-methylguanine	:	5.0 cm
Thymine	:	11.5 cm

(see sample chromatograms - Figs. 4 & 5)

Fig. 4. Radiochromatogram of a DNA hydrolysate developed by a modified two-stage technique in the same solvent showing separation of metabolically labelled normal and methylated purines.

(solvent: t-butanol - methyl ethyl ketone - conc. ammonia - water 40.30.20.10 v/v)

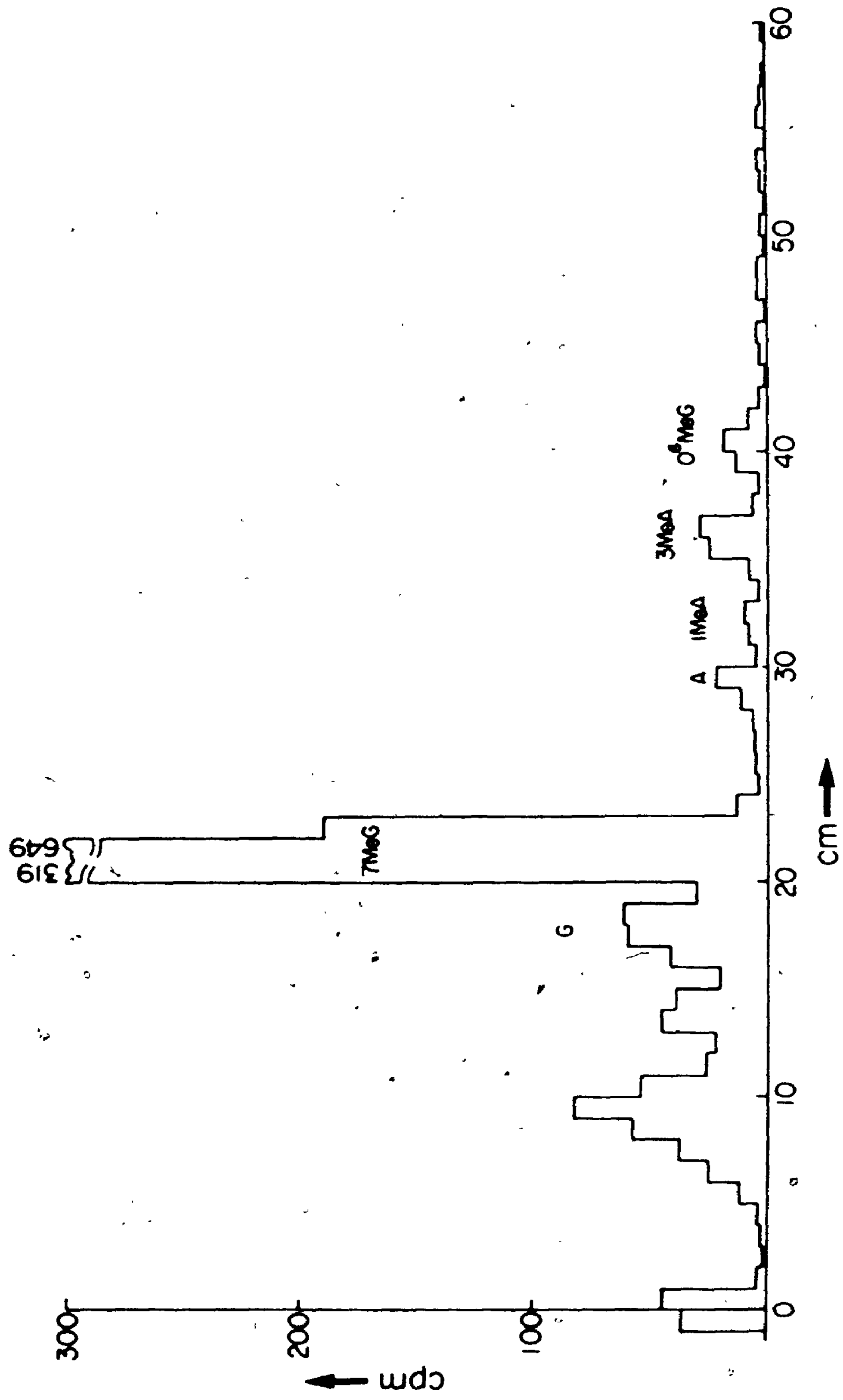


FIG. 4

Fig. 5. Re-chromatogram of the zone corresponding to 0^6-MeG in the two-stage chromatogram (ref. Fig. 4) showing its separation from the contaminants.
(solvent: upper phase of ethyl acetate - formic acid - water 60:5:35 v/v)

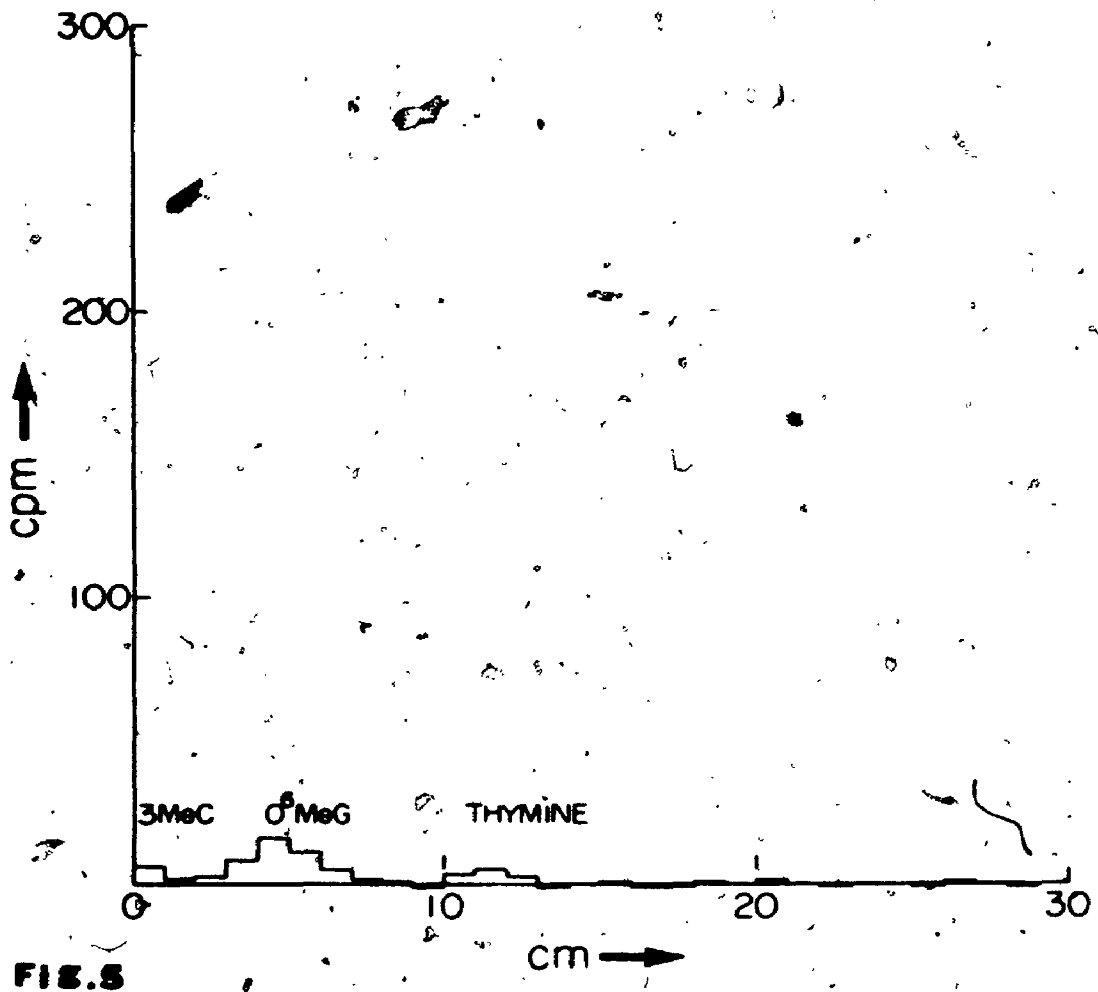


FIG. 5

Determination of the linearity of counts: With different dilutions of ^{14}C-toluene internal standard in 10 ml of the phosphor containing naphthalene (100 gm), 2,5-diphenyloxazole (4 gm), p-bis-2-5-phenyloxazolylbenzene (100 mg), methanol (100 ml) and dioxane (to 1 liter), the counts/min. as obtained with the counter used in this study were:

	Expected radioactivity	Observed radioactivity
Starting standard (initially diluted) - 10 ml	-	333.0
1 ml starting standard + 9 ml phosphor	33.0	33.2
2 ml starting standard + 8 ml phosphor	66.0	67.5
4 ml starting standard + 6 ml phosphor	132.0	135.6
6 ml starting standard + 4 ml phosphor	198.0	196.9
8 ml starting standard + 2 ml phosphor	264.0	276.0

Gross appearance of the organs and the yield of DNA from tissues: Following treatment with MMS, there were no observable changes either in the gross appearance of the organs or in their yields of DNA at any time point. Following treatment with MNUA, in contrast, observable changes were noted at 12 hours. The spleen and the thymus looked shrunken and their yields of DNA were less than usual while no DNA could be isolated from the bone marrow.

DNA alkylation: The results of the analyses are shown in Tables 5 to 24 and in Figures 6 to 24.

The first point to note is that with both agents, MNUA and MMS, a considerable amount of radioactivity (10 - 50%) has been relegated to

the category "other". In all instances, most of that radioactivity was present at the origin or had migrated less than guanine (see sample chromatogram - Fig. 4). It might represent metabolically labelled apurinic acid (O'Connor et al., 1973), or apurinic acid labelled by a methyl group at a phosphate (Bannon and Verly, 1972; Lawley, 1973) or on a pyrimidine, or RNA, or other impuities. No attempt was made to resolve this radioactivity further.

The second point to note is that with both agents metabolic labelling of guanine and adenine increased with time after treatment. It was detectable even at 0.3 hour and was more marked in tissues in which many cells were in active cell cycle (Figs. 13 - 15 and 22 - 24).

The third point to be made is that some of the products, although detectable as "peaks" in the chromatogram, were below the level of significance of 8 cpm as defined above (Tables 15 - 24). This was especially true of all the values shown for O^6-MeG following treatment with MMS. In other instances no "peaks" were seen.

The main observations may be described as follows:

In terms of the products:-

a) With both agents and in all tissues, the major methylation product was 7MeG which comprised about 85% of the methylpurines (Figs. 10 & 20).

b) The next most abundant product, in all cases, was 3MeA which when due to MMS comprised about 12 - 15% of the products (Fig. 21) and when due to MNUA about 10% of the products (Fig. 11).

c) O^6-MeG was generated in all tissues following treatment with MNUA and its contribution was about 4% (Fig. 12). Possibly, it was also generated in all tissues following

treatment with MMS but its level, in all instances, was below significance.

d) 1-MeA was also detected in all tissues following treatment with MMS (Table 10) and in some tissues (liver, lung and kidney) following treatment with MNUA (Table 7) but its level, too, in most cases, was below significance.

In terms of the agents:-

Following treatment with MMS-

a) Both 7MeG and 3MeA attained the highest level at 2 hours in all tissues except lung where it was attained at 1 hour (Figs. 17 & 18).

b) After attaining the peak points, the product levels continuously declined (Figs. 17 & 18).

c) With a possible exception of bone marrow and spleen which showed slightly higher levels of 3MeA, no major differences between tissues were observed in product levels (Figs. 17 & 18).

d) Tissues were found to differ from each other in the differential removal of 3MeA relative to the bulk product 7MeG (Figs. 20 & 21). The differential removal was most marked in liver and kidney, least marked in bone marrow and spleen, and intermediate in lung, thymus and gut.

e) The overall patterns of alkylation were thus quite similar in all tissues; the level of alkylation rose gradually, reached a peak at 2 hours and then declined over the course of 12 hours (Fig. 16).

Following treatment with MNUA -

a) Although the level of 7MeG appeared to reach a maximum at 4.5 hours and a minor second peak at 0.3 hours in most tissues (Fig. 7), the significance and reproducibility of either peak could not be demonstrated. 3MeA and O^6-MeG also appeared to show two peaks in most tissues (Figs. 8 & 9), but again, the significance and reproducibility of the two peaks could not be established.

b) Marked differences in 7MeG levels were observed between tissues at all time points; the tissues appeared to be divided into two groups - high level group which included gut, liver and kidney while the rest of the tissues were included in the low level group (Fig. 7).

c) Tissue differences in the differential removal of 3MeA (Fig. 11) similar to those seen after MMS were suggested but could not be proven from the data.

d) As with 7MeG, marked differences in the levels of O^6-MeG were observed between tissues and the same type of division was apparent - gut, liver and kidney falling into the high level group while the rest falling into the low level group (Fig. 9).

e) As apparent, the overall patterns of alkylation showed variations between tissues and to some extent between products in contrast to the lack of such variations following treatment with MMS.

TABLE 5

DNA ALKYLATION AT 0.3 HOUR FOLLOWING 0.6 MOLE MNUA/KG

Percentage of DNA base alkylated
(Relative percentage of the individual products in parentheses).

Tissue	Total	7MeG	3MeA	O^6MeG	1MeA
Gut	0.0230	0.0941 (90.2)	0.0063 (7.7)	0.0021 (2.0)	---+
Liver	0.0391	0.1593 (89.7)	0.0086 (6.2)	0.0057 (3.2)	0.0011 (0.8)
Spleen	0.0145	0.0545 (82.5)	0.0068 (13.1)	0.0029 (4.4)	---+
Thymus	0.0128	0.0501 (85.9)	0.0048 (10.5)	0.0021 (3.6)	---+
Bone Marrow	0.0134	0.0489 (80.2)	0.0066 (13.8)	0.0037 (6.0)	---+
Lung	0.0143	0.0564 (87.1)	0.0046 (9.0)	0.0025 (3.9)	---+
Kidney	0.0201	0.0779 (85.1)	0.0089 (12.4)	0.0023 (2.5)	---+

+ no peak seen in radiochromatogram

TABLE 6

DNA ALKYLATION AT 1.0 HOUR FOLLOWING 0.6 M MOLE MNUA/KG

Percentage of DNA base alkylated

(Relative percentage of the individual products in parentheses)

Tissue	Total	7MeG	3MeA	O^6MeG	1MeA
Gut	0.0271	0.1123 (91.0)	0.0055 (5.7)	0.0039 (3.2)	---+
Liver	0.0349	0.1431 (90.1)	0.0066 (5.3)	0.0048 (3.0)	0.0019 (2.5)
Spleen	0.0136	0.0537 (87.1)	0.0047 (9.7)	0.0020 (3.2)	---+
Thymus	0.0094	0.0353 (82.7)	0.0045 (13.4)	0.0016 (3.8)	---+
Bone marrow	0.0118	0.0452 (84.3)	0.0044 (10.4)	0.0028* (5.2)	---+
Lung	0.0192	0.0774 (88.4)	0.0059 (8.6)	0.0025 (2.9)	---+
Kidney	0.0198	0.0803 (89.3)	0.0054 (7.7)	0.0018 (2.0)	0.0007* (1.0)

* cpm below level of significance, though peak seen in radiochromatogram

+ no peak seen in radiochromatogram

TABLE 7

DNA ALKYLATION AT 2.0 HOURS FOLLOWING 0.6 M MOLE MNUA/KG

Percentage of DNA base alkylated

(Relative percentage of the individual products in parentheses).

Tissue	Total	7MeG	3MeA	O^6MeG	1MeA
Gut	0.0288	0.1193 (91.0)	0.0062 (6.0	0.0038 (2.9)	---+
Liver	0.0365	0.1488 (89.6)	0.0085 (6.5)	0.0050 (3.0)	0.0012 (0.9)
Spleen	0.0180	0.0705 (86.1)	0.0067 (10.5)	0.0027 (3.3)	---+
Thymus	0.0131	0.0524 (87.7)	0.0046 (9.9)	0.0014 (2.4)	---+
Bone marrow	0.0146	0.0552 (83.5)	0.0056 (10.7)	0.0038 (5.8)	---+
Lung	0.0154	0.0599 (85.4)	0.0058 (10.5)	0.0024 (3.4)	0.0003* (0.6)
Kidney	0.00254	0.1026 (88.9)	0.0074 (8.2)	0.0025 (2.2)	0.0006* (0.7)

* cpm below level of significance, though peak seen in radiochromatogram

+ no peak seen in radiochromatogram

TABLE 8

DNA ALKYLATION AT 4.5 HOURS FOLLOWING 0.6 M MOLE MNUA/KG

Percentage of DNA base alkylated

(Relative percentage of the individual products in parentheses)

Tissue	Total	7MeG	3MeA	O^6MeG	1MeA
Gut	0.0327	0.1325 (89.1)	0.0060 (5.1)	0.0086 (5.8)	---+
Liver	0.0610	0.2542 (91.7)	0.0087 (4.0)	0.0094 (3.4)	0.0017 (0.8)
Spleen	0.0177	0.0691 (86.1)	0.0052 (8.3)	0.0044 (5.5)	---+
Thymus	0.0187	0.0736 (86.6)	0.0063 (9.5)	0.0033 (3.9)	---+
Bone marrow	0.0149	0.0538 (79.4)	0.0063 (11.9)	0.0059* (8.7)	---+
Lung	0.0225	0.0900 (87.8)	0.0052 (6.5)	0.0051 (5.0)	0.0006* (0.7)
Kidney	0.0285	0.1131 (87.3)	0.0046 (4.5)	0.0097 (7.5)	0.0007* (0.7)

* cpm below level of significance, though peak seen in radiochromatogram

+ no peak seen in radiochromatogram

TABLE 9

DNA ALKYLATION AT 12 HOURS FOLLOWING 0.6 M MOLE MNUA/KG

Percentage of DNA base alkylated

(Relative percentage of the individual products in parentheses)

Tissue	Total	7MeG	3MeA	O^6MeG	1MeA
Gut	0.0225	0.0918 (89.8)	0.0052 (6.5)	0.0037 (3.6)	---+
Liver	0.0346	0.1451 (92.3)	0.0048 (3.9)	0.0046 (2.9)	0.0011 (0.9)
Spleen	0.0130	0.0511 (86.6)	0.0050 (10.8)	0.0016* (2.7)	---+
Thymus	0.0147	0.0600 (90.0)	0.0039 (7.4)	0.0017 (2.6)	---+
Bone marrow	DNA could not be isolated				
Lung	0.0126	0.0514 (89.5)	0.0032 (7.0)	0.0019* (3.4)	---+
Kidney	0.0188	0.0770	0.0039	0.0026	0.0007*

* cpm below level of significance, though peak seen in radiochromatogram

+ no peak seen in radiochromatogram

TABLE 10

DNA ALKYLATION AT 0.3 HOURS FOLLOWING 1.2 M MOLE MMS/KG

Percentage of DNA base alkylated

(Relative percentage of the individual products in parentheses)

Tissue	Total	7MeG	3MeA	0^6MeG	1MeA
Gut	0.0095	0.0368 (85.6)	0.0040 (12.0)	0.0005* (1.1)	0.0004* (1.3)
Liver	0.0134	0.0509 (83.8)	0.0049 (10.3)	0.0005* (0.9)	0.0024 (5.0)
Spleen	0.0109	0.0436 (87.7)	0.0045 (11.5)	0.0002* (0.5)	0.0001* (0.3)
Thymus	0.0089	0.0346 (85.0)	0.0042 (13.1)	0.0002* (0.6)	0.0004* (1.2)
Bone marrow	0.0079	0.0294 (81.9)	0.0044 (15.6)	0.0003* (0.9)	0.0004* (1.6)
Lung	0.0090	0.0345 (84.6)	0.0043 (13.5)	0.0002* (0.5)	0.0004* (1.4)
Kidney	0.0094	0.0365 (85.7)	0.0042 (12.6)	0.0002* (0.6)	0.0003* (1.0)

* cpm below level of significance, though peak seen in radiochromatogram

TABLE 11

DNA ALKYLATION AT 1 HOUR FOLLOWING 1.2 M MOLE MMS/KG

Percentage of DNA base alkylated

(Relative percentage of the individual products in parentheses)

Tissue	Total	7MeG	3MeA	O^6MeG	1MeA
Gut	0.0154	0.0583 (83.4)	0.0070 (12.8)	0.0010* (1.4)	0.0013 (2.3)
Liver	0.0156	0.0616 (86.5)	0.0063 (11.2)	0.0006* (0.8)	0.0008* (1.4)
Spleen	0.0162	0.0647 (87.9)	0.0067 (11.6)	0.0004* (0.5)	---+
Thymus	0.0164	0.0633 (85.0)	0.0073 (12.4)	0.0007* (1.0)	0.0008* (1.4)
Bone marrow	0.0132	0.0513 (85.2)	0.0065 (13.8)	0.0006* (1.0)	---+
Lung	0.0189	0.0720 (83.7)	0.0097 (14.4)	0.0007* (0.8)	0.0007* (1.0)
Kidney	0.0158	0.0620 (86.1)	0.0065 (11.5)	0.0006* (0.8)	0.0008 (1.5)

* cpm below level of significance, though peak seen in radiochromatogram

+ no peak seen in radiochromatogram

TABLE 12

DNA ALKYLATION AT 2.0 HOURS FOLLOWING 1 2 M MOLE MMS/KG

Percentage of DNA base alkylated

(Relative percentage of the individual products in parentheses)

Tissue	Total	7MeG	3MeA	O^6MeG	1MeA
Gut	0.0205	0.0818 (87.8)	0.0085 (11.6)	0.0006* (0.6)	---+
Liver	0.0169	0.0678 (88.4)	0.0066 (11.0)	0.0004* (0.5)	---+
Spleen	0.0186	0.0731 (86.5)	0.0087 (13.1)	0.0003* (0.4)	---+
Thymus	0.0171	0.0670 (86.2)	0.0083 (13.6)	0.0002* (0.2)	---+
Bone marrow	0.0143	0.0537 (82.6)	0.0087 (17.0)	0.0003* (0.4)	---+
Lung	0.0178	0.0701 (86.6)	0.0082 (12.9)	0.0004* (0.5)	---+
Kidney	0.0177	0.0704	0.0075	0.0003*	---+

* cpm below level of significance, though peak seen in radiochromatogram

+ no peak seen in radiochromatogram

TABLE 13

DNA ALKYLATION AT 4.5 HOURS FOLLOWING 1.2 M MOLE MMS/KG

Percentage of DNA base alkylated

(Relative percentage of the individual products in parentheses)

Tissue	Total	7MeG	3MeA	0^6MeG	1MeA
Gut	0.0098	0.0398 (89.4)	0.0034 (9.6)	0.0004* (1.0)	---+
Liver	0.0115	0.0489 (93.8)	0.0025 (6.1)	---+	---+
Spleen	0.0111	0.0426 (84.1)	0.0062 (15.5)	0.0002* (0.4)	---+
Thymus	0.0104	0.0410 (86.7)	0.0049 (13.3)	---+	---+
Bone marrow	0.0094	0.0336 (78.6)	0.0071 (21.1)	0.0001* (0.2)	---+
Lung	0.0096	0.0382 (87.5)	0.0042 (12.2)	0.0001* (0.3)	---+
Kidney	0.0106	0.0450 (92.9)	0.0027 (7.1)	---+	---+

* cpm below level of significance, though peak seen in radiochromatogram

\+ no peak seen in radiochromatogram

TABLE 14

DNA ALKYLATION AT 12 HOURS FOLLOWING 1.2 M MOLE MMS/KG

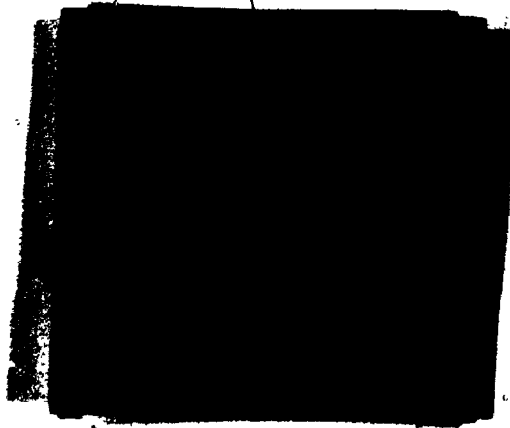
Percentage of DNA base alkylated

(Relative percentage of the individual products in parentheses)

Tissue	Total	7MeG	3MeA	O^6MeG	1MeA
Gut	0.0073	0.0297 (89.4)	0.0028 (10.6)	---+	---+
Liver	0.0104	0.0444 (94.0)	0.0022 (6.0)	---+	---+
Spleen	0.0092	0.0351 (84.0)	0.0053 (16.0)	---+	---+
Thymus	0.0084	0.0341 (89.1)	0.0033 (10.9)	---+	---+
Bone marrow	0.0066	0.0241 (80.7)	0.0045 (19.3)	---+	---+
Lung	0.0075	0.0302 (88.9)	0.0030 (11.1)	---+	---+
Kidney	0.0093	0.0395 (93.5)	0.0022 (6.5)	---+	---+

+ no peak seen in radiochromatogram

2 OF/DE 3



ARC · 374

TABLE 15

DNA - 0.3 hour after MNUA

Balance Sheet (cpm)

Tissue	mgm chromato-graphed	G	A	7MeG	3MeA	0^6MeG	1MeA	Other	Total
Gut	0.46	58	22	539	46	12	0+	327	1004
Liver	0.46	100	13	1013	71	36	9	369	1611
Spleen	0.38	58	73	301	48	16	0+	234	730
Thymus	0.58	27	7*	356	43	15	0+	150	598
Bone marrow	0.20	22	21	106	18	8	0+	144	319
Lung	0.25	11	5*	189	20	9	0+	50	284
Kidney	0.58	10	7*	561	82	16	0+	137	813

* Below level of significance, though peak seen in radiochromatogram

+ No peak seen in radiochromatogram

TABLE 16

DNA - 1.0 hour after MNUA

Balance Sheet (cpm)

Tissue	mgm chromato-graphed	G	A	7MeG	3MeA	0^6MeG	1MeA	Other	Total
Gut	0.55	260	75	809	51	29	0+	505	1729
Liver	0.61	163	44	1216	72	40	21	556	2112
Spleen	0.77	208	287	554	62	20	0+	609	1740
Thymus	0.58	30	51	337	55	16	0+	275	764
Bone marrow	0.13	68	98	73	9	4*	0+	145	397
Lung	0.63	18	3*	465	45	15	0+	18	564
Kidney	0.65	37	14	617	53	13	7*	208	949

* Below level of significance, though peak seen in radiochromatogram

+ No peak seen in radiochromatogram

TABLE 17

DNA - 2.0 hours after MNUA

Balance Sheet (cpm)

Tissue	mgm chromato-graphed	G	A	7MeG	3MeA	0^6MeG	1MeA	Other	Total
Gut	0.57	399	239	1015	67	33	0+	865	2618
Liver	0.52	68	26	1179	85	40	12	724	2134
Spleen	0.76	448	684	843	103	33	0+	1225	3336
Thymus	0.96	78	171	724	82	20	0+	361	1436
Bone marrow	0.43	429	721	366	47	25	0+	963	2551
Lung	0.26	16	13	319	39	13	2*	193	595
Kidney	0.52	34	7*	815	75	21	6*	272	1230

* Below level of significance, though peak seen in radiochromatogram

+ No peak seen in radiochromatogram

TABLE 18

DNA - 4.5 hours after MNUA

Balance Sheet (cpm)

Tissue	mgm chromato-graphed	G	A	7MeG	3MeA	0^6MeG	1MeA	Other	Total
Gut	0.49	586	340	805	46	52	0+	938	2767
Liver	0.48	230	135	1557	68	57	14	1650	3711
Spleen	0.42	81	157	390	38	25	0+	432	1123
Thymus	0.54	30	162	462	50	21	0+	332	1057
Bone marrow	0.09	147	318	64	10	7*	0+	335	881
Lung	0.55	34	18	578	43	33	5*	122	833
Kidney	0.66	87	55	915	47	78	7*	355	1545

* Below level of significance, though peak seen in radiochromatogram

+ No peak seen in radiochromatogram

TABLE 19

DNA - 12 hours after MNUA

Balance Sheet (cpm)

Tissue	mgm chromato-graphed	G	A	7MeG	3MeA	O^6MeG	1MeA	Other	Total
Gut	0.82	865	1173	1125	82	46	0+	1022	4313
Liver	0.44	63	44	973	41	30	9	441	1601
Spleen	0.10	46	89	72	9	2*	0+	326	544
Thymus	0.63	20	144	551	45	16	0+	358	1134
Bone marrow	No DNA could be isolated								
Lung	0.11	11	8	124	10	5*	0+	66	224
Kidney	0.36	41	24	492	31	17	6*	317	928

* Below level of significance, though peak seen in radiochromatogram

\+ No peak seen in radiochromatogram

TABLE 20

DNA - 0.3 hours after MMS

Balance Sheet (cpm)

Tissue	mgm chromato-graphed	G	A	7MeG	3MeA	0^6MeG	1MeA	Other	Total
Gut	0.72	19	8	411	58	5*	6*	139	646
Liver	0.58	207	10	380	47	4*	23	168	839
Spleen	0.72	16	8	377	49	2*	1*	26	479
Thymus	0.86	12	2	377	59	3*	5*	46	504
Bone marrow	0.05	6*	11	26	5	<1*	<1*	60	108
Lung	0.41	8	1*	215	34	1*	3*	40	302
Kidney	0.76	10	0+	365	54	3*	4*	48	484

* Below level of significance, though peak seen in radiochromatogram

+ No peak seen in radiochromatogram

TABLE 21

DNA - 1.0 hour after MMS

Balance Sheet (cpm)

Tissue	mgm chromato-graphed	G	A	7MeG	3MeA	0^6MeG	1MeA	Other	Total
Gut	0.50	52	32	362	56	6*	10	212	730
Liver	0.52	17	5*	343	45	3*	6*	45	467
Spleen	0.58	79	87	366	48	2*	0+	239	821
Thymus	0.59	14	15	380	55	5*	6*	84	559
Bone marrow	0.35	44	53	155	25	2*	0+	88	367
Lung	0.70	19	3*	459	79	5*	6*	98	669
Kidney	0.65	21	7*	444	59	4*	8	96	639

* Below level of significance, though peak seen in radiochromatogram

+ No peak seen in radiochromatogram

TABLE 22

DNA - 2.0 hours after MMS

Balance Sheet (cpm)

Tissue	mgm chromatographed	G	A	7MeG	3MeA	0^6MeG	1MeA	Other	Total
Gut	0.53	140	53	457	60	3*	0+	85	798
Liver	0.77	80	24	663	83	4*	0+	174	1028
Spleen	0.70	237	255	567	86	2*	0+	601	1748
Thymus	0.89	38	45	743	117	2*	0+	95	1040
Bone marrow	0.27	61	109	132	27	1*	0+	112	442
Lung	0.70	25	6*	534	79	3*	0+	85	732
Kidney	0.81	27	9	718	97	3*	0+	64	918

* Below level of significance, though peak seen in radiochromatogram

+ No peak seen in radiochromatogram

TABLE 23

DNA - 4.5 hours after MMS

Balance Sheet (cpm)

Tissue	mgm chromato-graphed	G	A	7MeG	3MeA	O^6MeG	1MeA	Other	Total
Gut	0.93	747	509	543	58	6*	0+	821	2684
Liver	0.43	193	51	258	17	0+	0+	240	759
Spleen	0.44	416	706	240	44	1*	0+	1061	2468
Thymus	0.66	42	127	360	55	0+	0+	230	814
Bone marrow	0.35	311	633	153	41	<1*	0+	812	1950
Lung	0.33	15	14	195	27	1*	0+	212	464
Kidney	0.47	26	15	264	20	0+	0+	155	480

* Below level of significance, though peak seen in radiochromatogram

+ No peak seen in radiochromatogram

TABLE 24

DNA - 12 hours after MMS

Balance Sheet (cpm)

Tissue	mgm chromato-graphed	G	A	7MeG	3MeA	0^6MeG	1MeA	Other	Total
Gut	0.83	823	1008	344	41	0^+	0^+	1453	3669
Liver	0.63	223	134	390	25	0^+	0^+	1830	2602
Spleen	0.52	427	796	238	45	0^+	0^+	1351	2857
Thymus	1.02	42	168	416	51	0^+	0^+	306	983
Bone marrow	0.20	274	535	67	16	0^+	0^+	584	1476
Lung	0.33	27	32	175	22	0^+	0^+	99	355
Kidney	0.61	80	47	340	24	0^+	0^+	161	652

+ No peak seen in radiochromatogram

Fig. 6. Methylation of DNA in various mouse organs by (^{14}C)-methyl-nitrosourea (dose: 0.6 mM/Kg body weight) - expressed as molar percent of total bases.
(Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

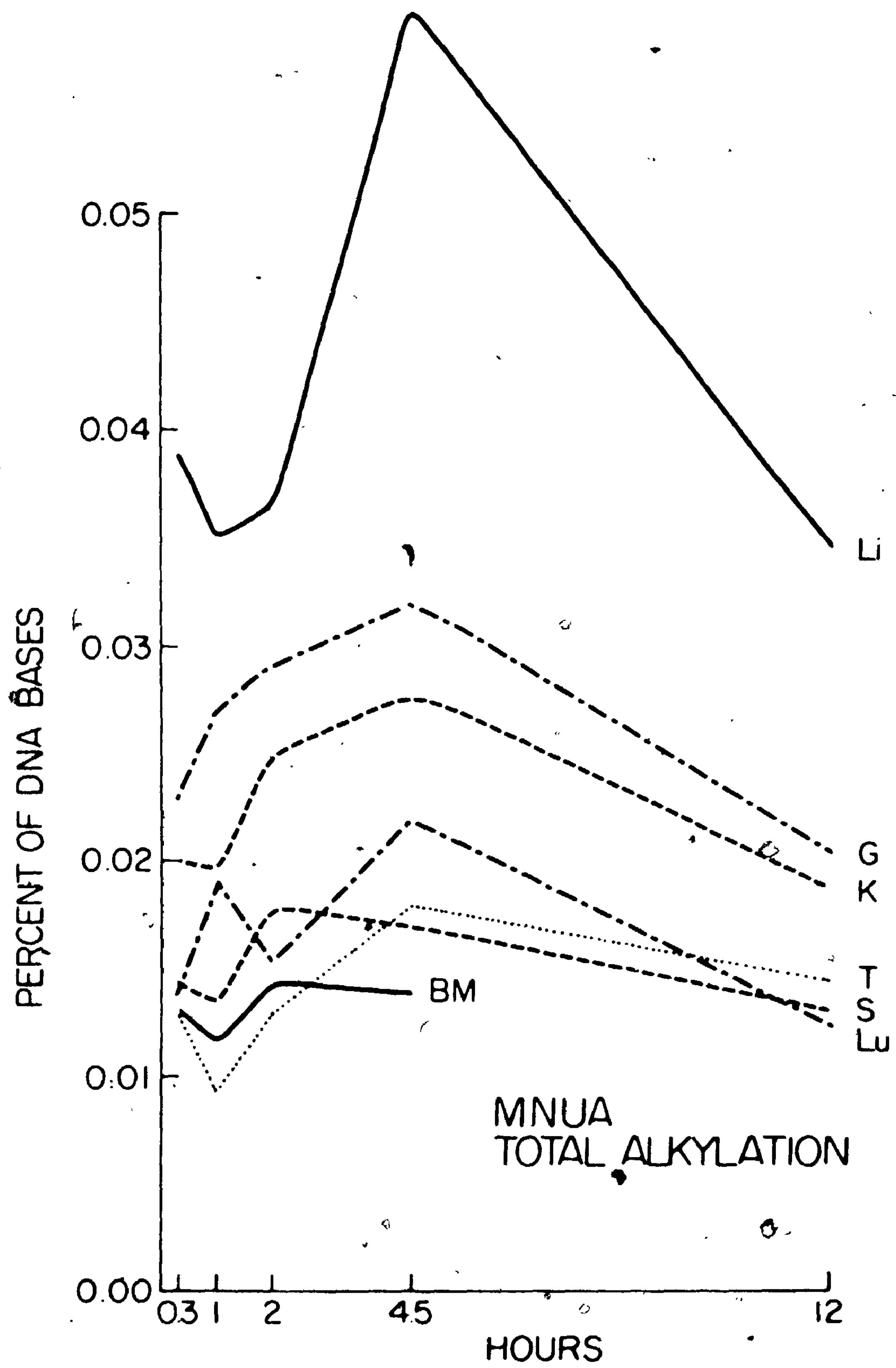


Fig. 6

Fig. 7. Molar percent of guanine methylated as 7MeG in the DNA of different mouse tissues following treatment with (^{14}C)-methylnitrosourea (dose: 0.6 mM/Kg body weight). (Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

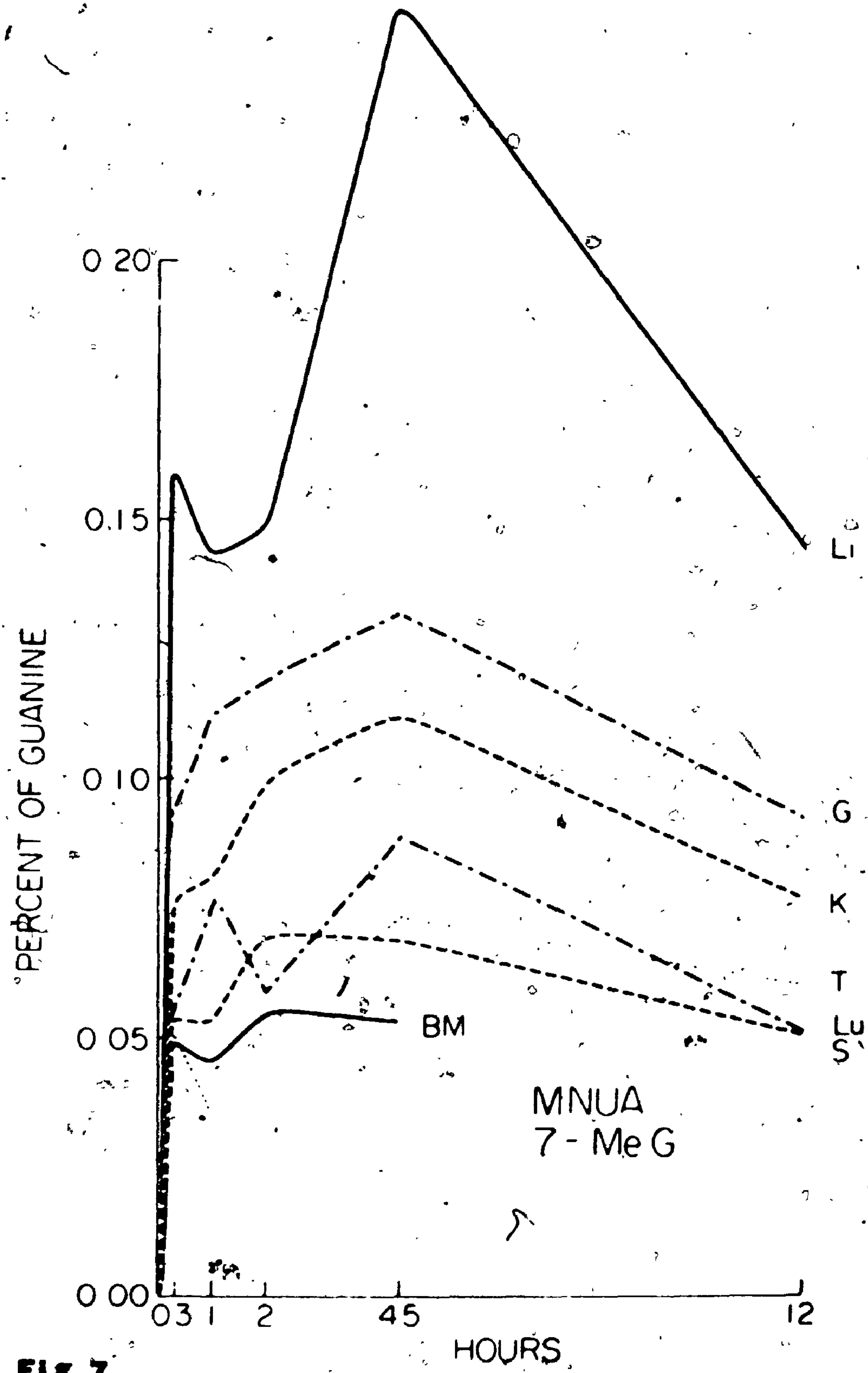


Fig.7

Fig. 8. Molar percent of adenine methylated as 3MeA in the DNA of different mouse tissues following treatment with (^{14}C)-methylnitrosourea (dose; 0.6 mM/Kg body weight). (Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

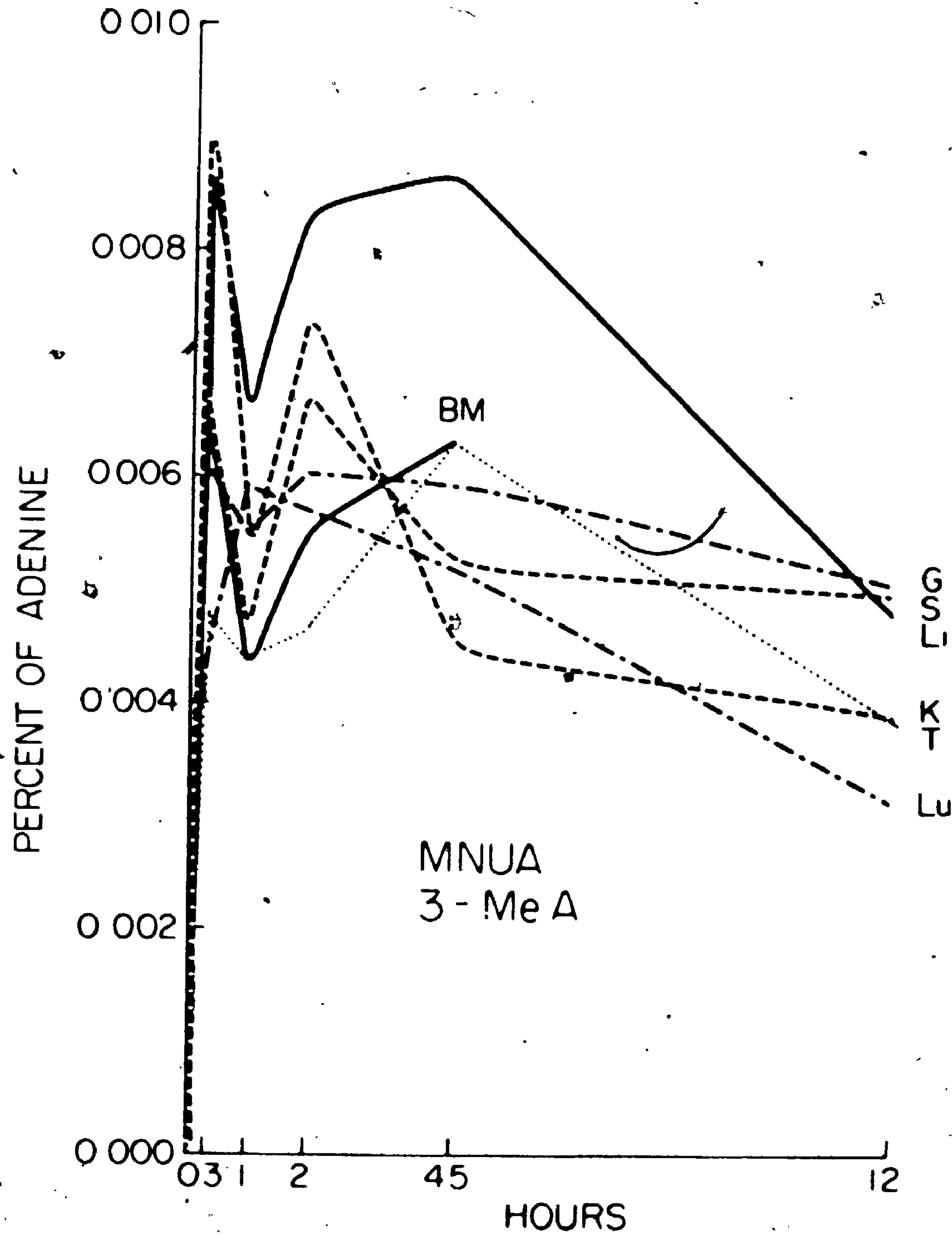


FIG. 8

Fig. 9. Molar percent of guanine methylated as O^6-MeG in the DNA of different mouse tissues following treatment with (^{14}C)-methylnitrosourea (dose: 0.6 mM/Kg body weight). (Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

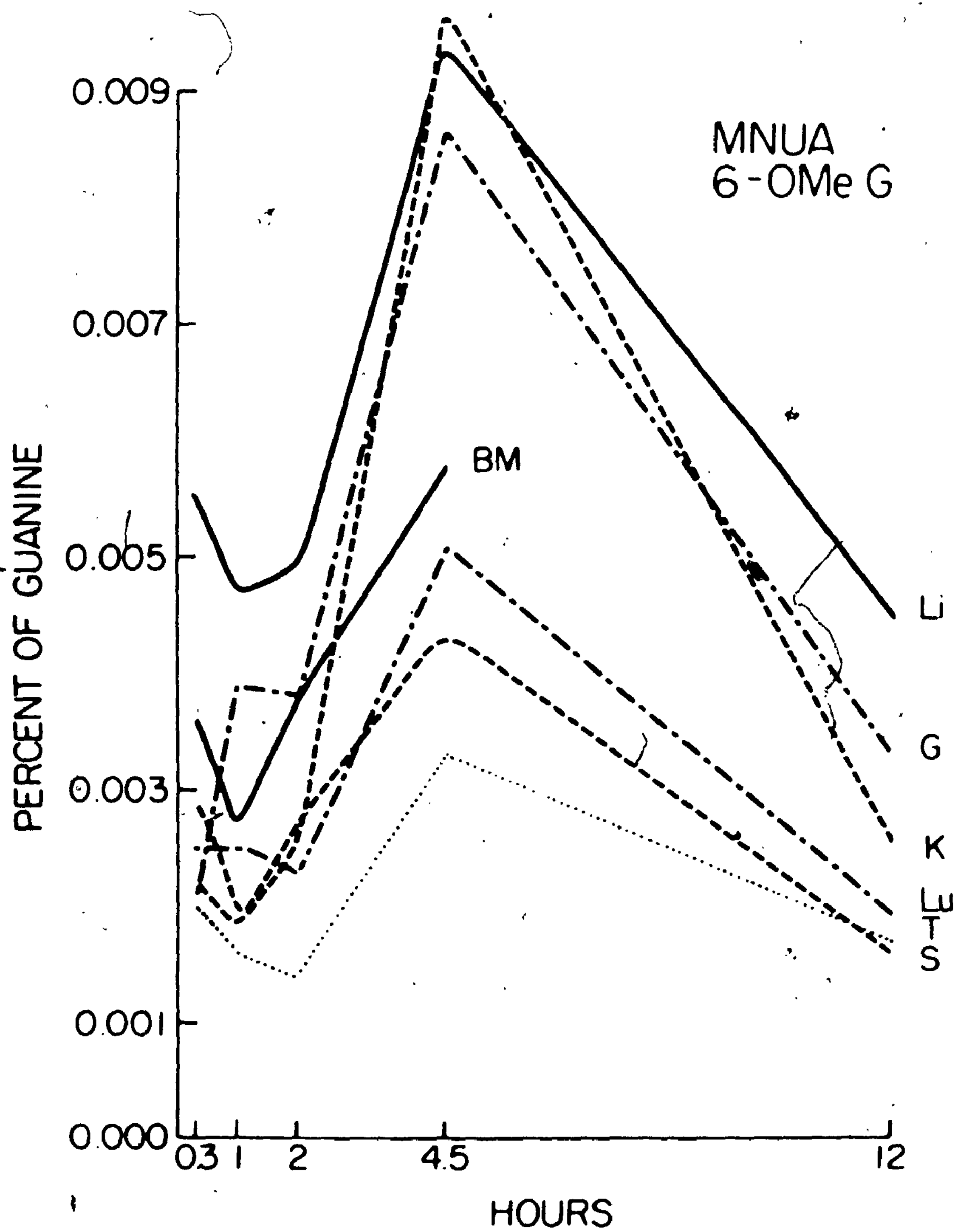


FIG.9

Fig. 10. Proportion of 7MeG in the DNA of different mouse tissues expressed as percent of total radioactivity due to methylated purines following treatment with (^{14}C)-methylnitrosourea (dose: 0.6 mM/Kg body weight).
(Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

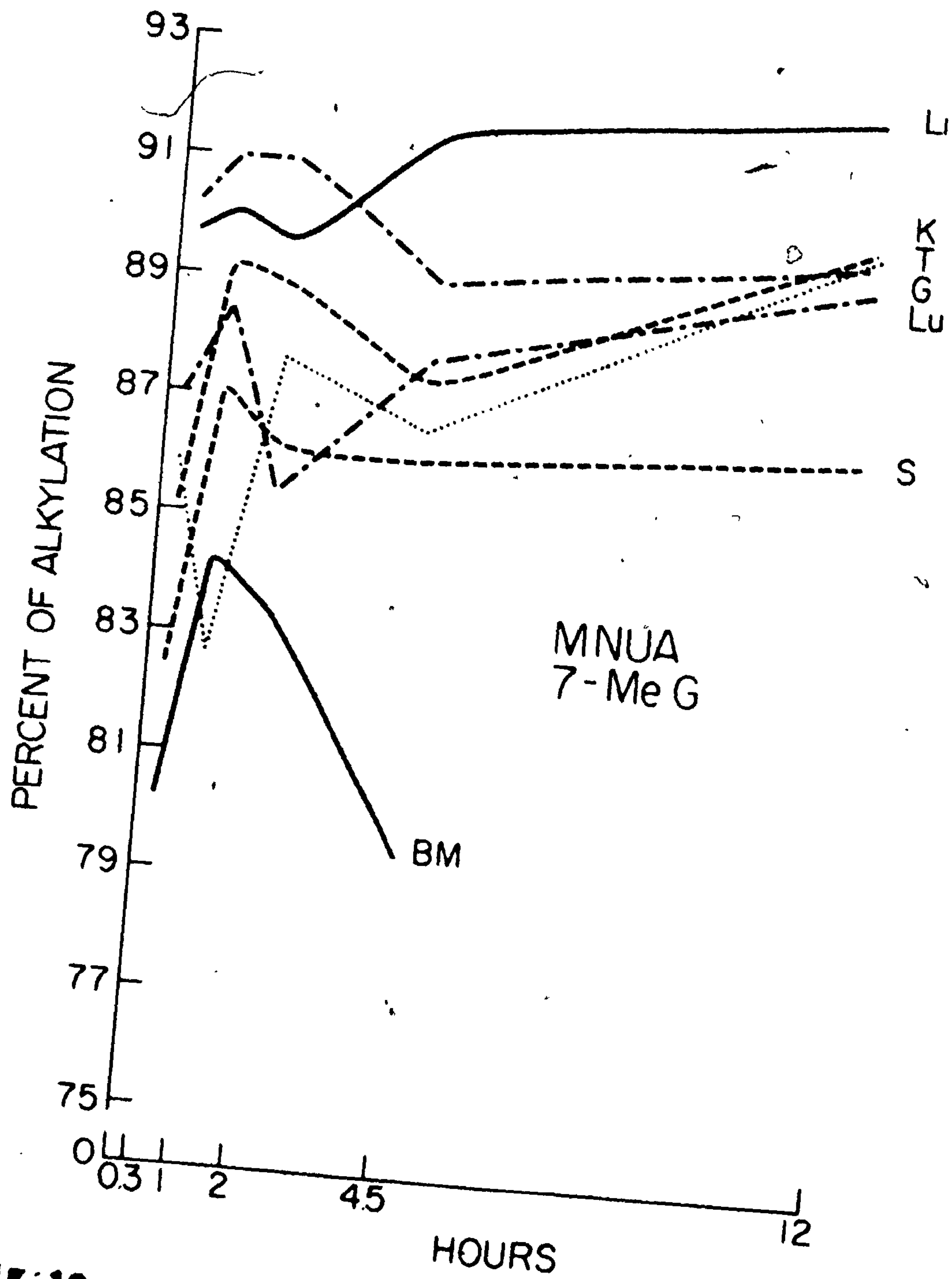


FIG: 10

Fig. 11. Proportion of 3MeA in the DNA of different mouse tissues expressed as percent of total radioactivity due to methylated purines following treatment with (^{14}C)-methylnitrosourea (dose: 0.6 mM/Kg body weight).
(Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

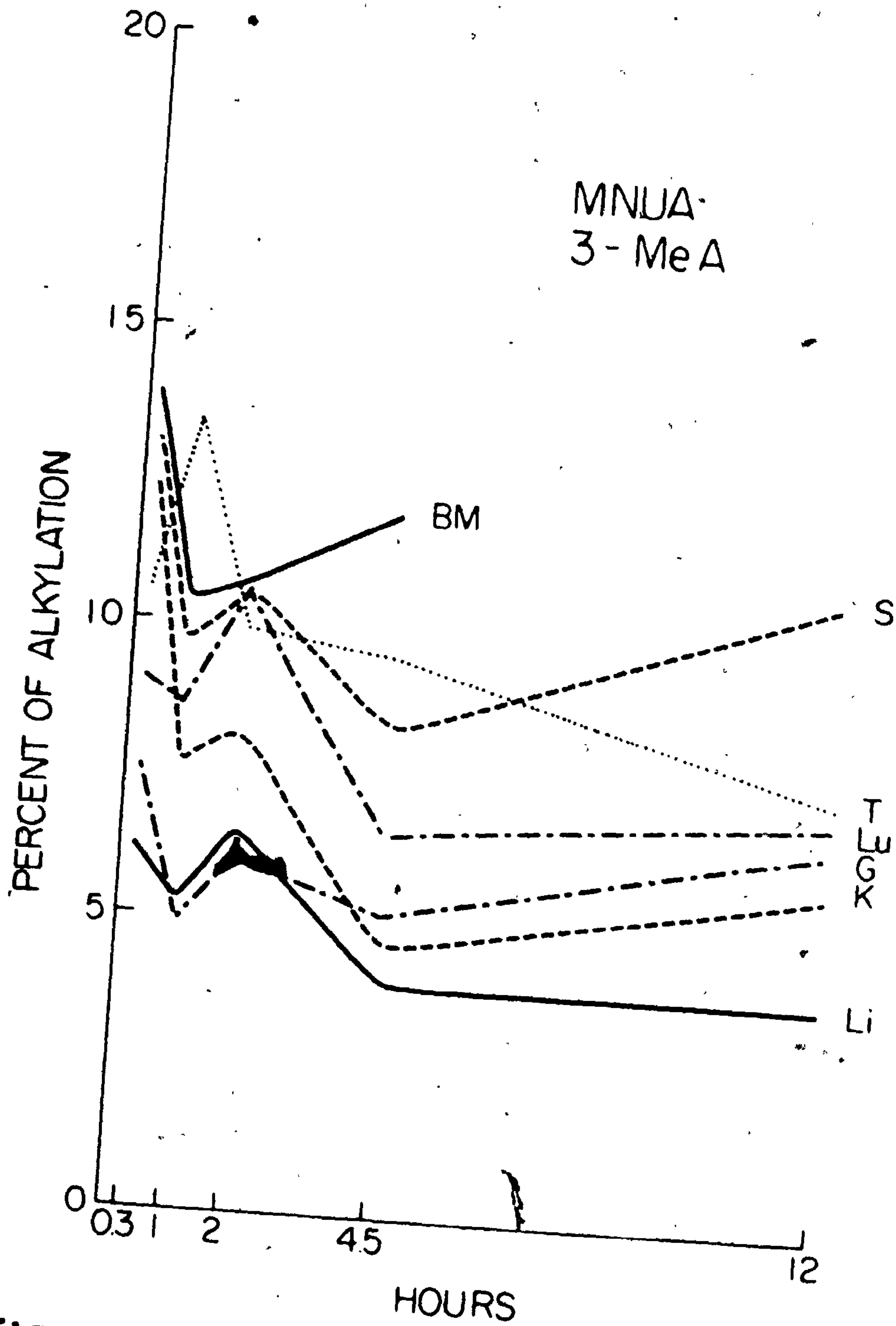


Fig. 11

Fig. 12. Proportion of 0^6-MeG in the DNA of different mouse tissues expressed as percent of total radioactivity due to methylated purines following treatment with (^{14}C)-methylnitrosourea (dose: 0.6 mM/Kg body weight).
(Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

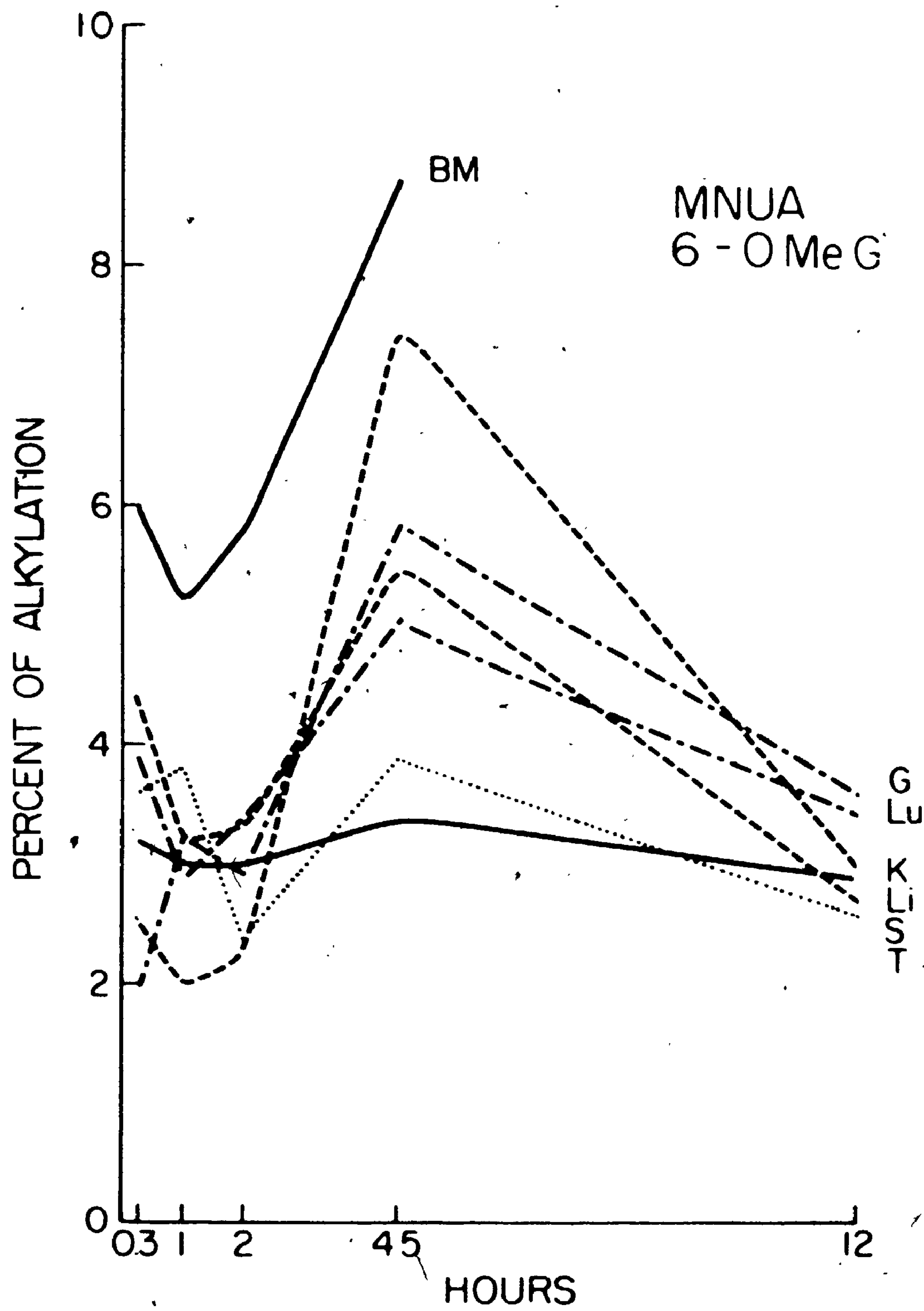


Fig. 12

Fig. 13. Metabolic incorporation of labelled carbon (^{14}C) into guanine of DNA in different mouse tissues following treatment with (^{14}C)-methylnitrosourea (dose; 0.6 mM/Kg body weight).

(Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

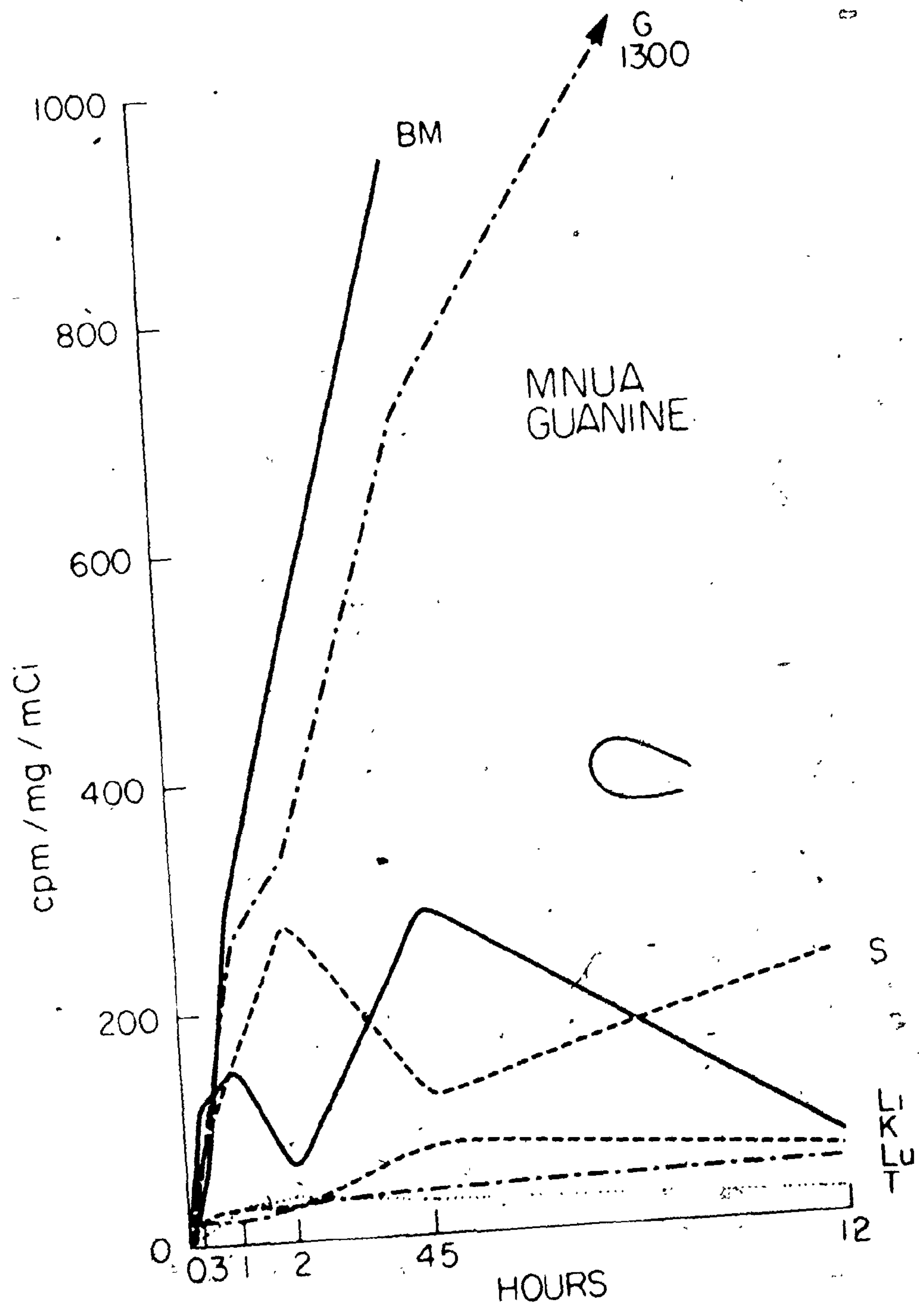


FIG. 13

Fig. 14. Metabolic incorporation of labelled carbon (^{14}C) into adenine of DNA in different mouse tissues following treatment with (^{14}C)-methylnitrosourea (dose: 0.6 mM/Kg body weight).

(Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

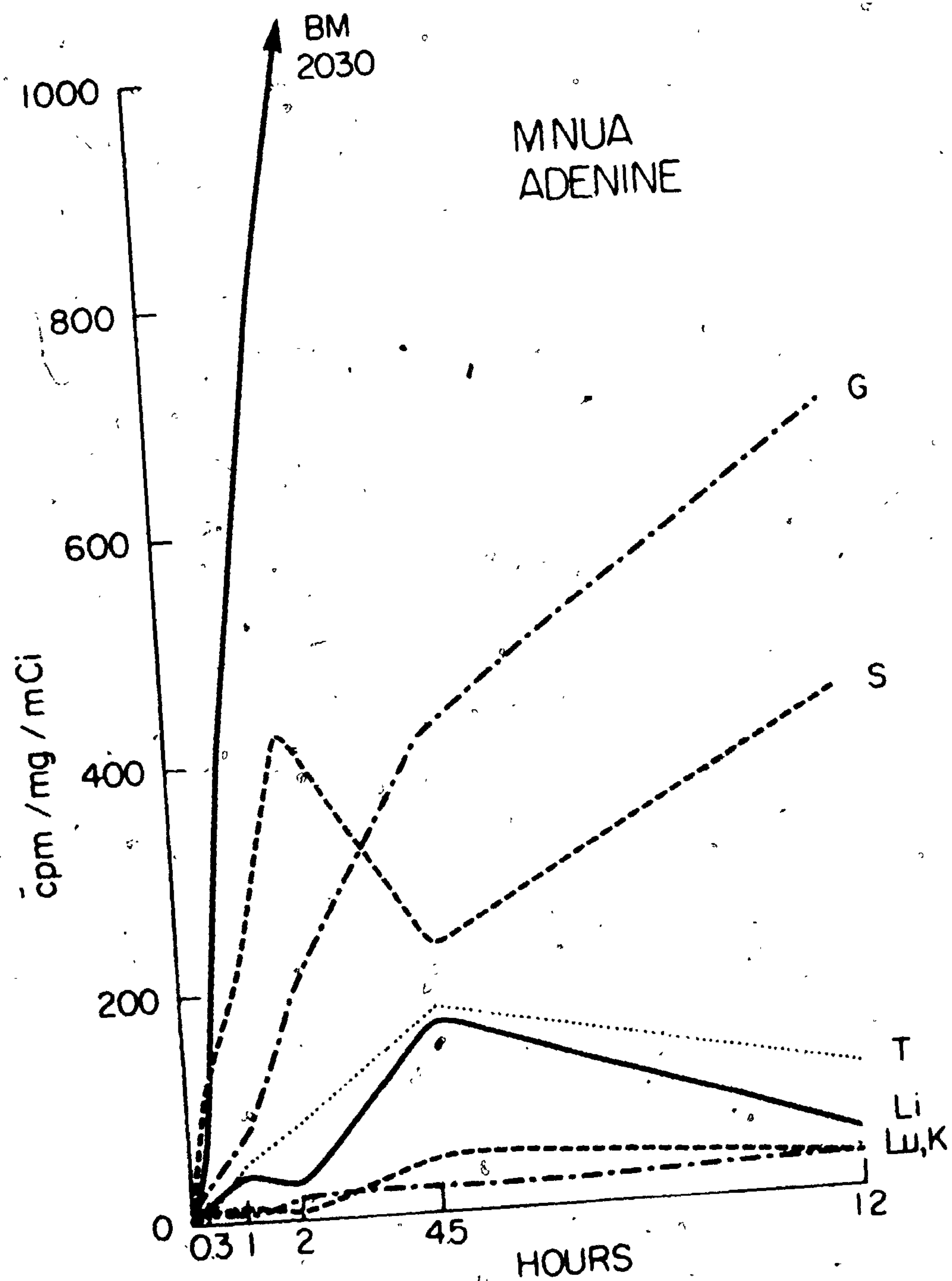


FIG.14

Fig. 15. Metabolically labelled guanine/adenine ratio in the DNA of different mouse tissues following treatment with (^{14}C)-methylnitrosourea (dose: 0.6 mM/Kg body weight) (after normalization).

(Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

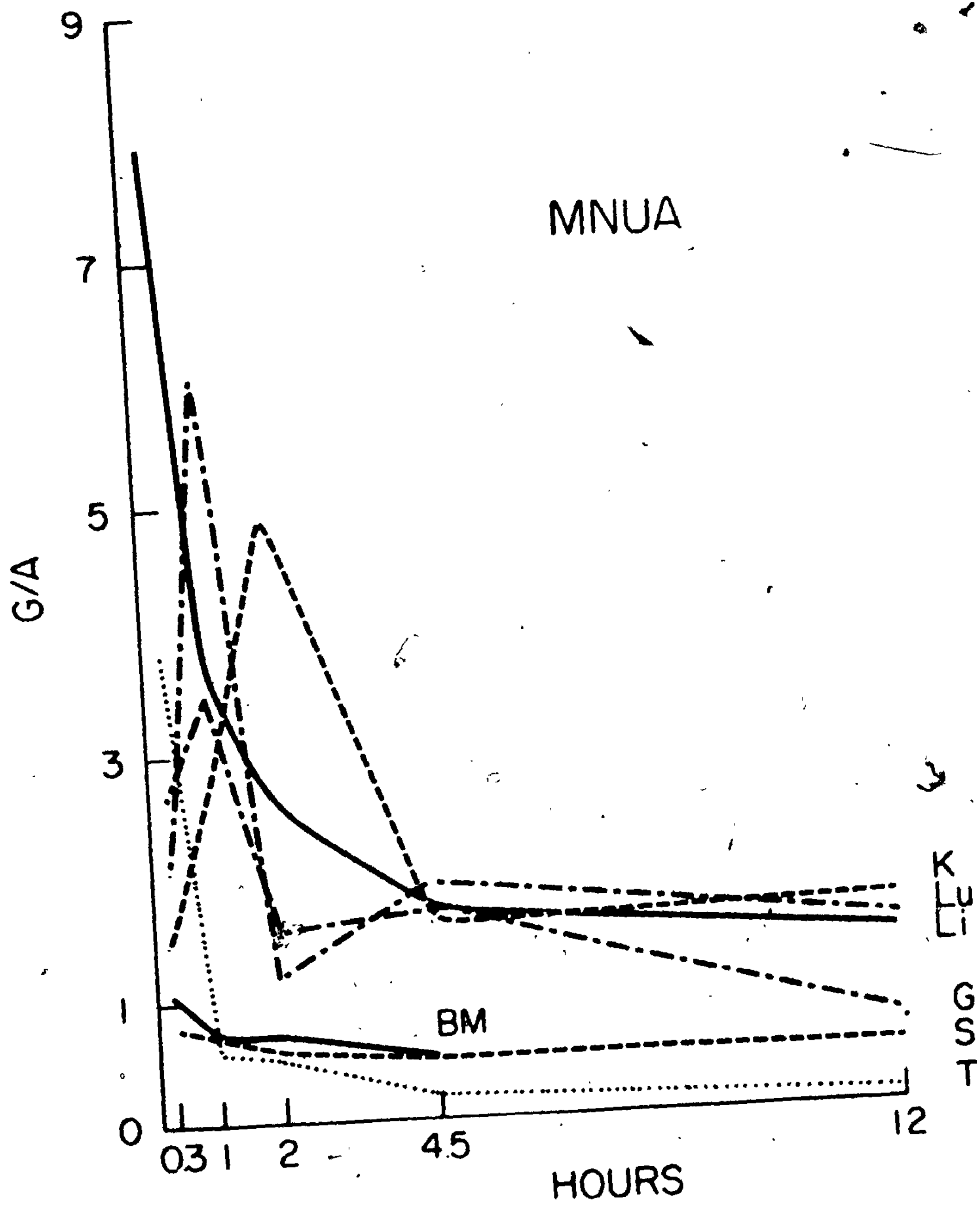


Fig.15

Fig. 16. Methylation of DNA in various mouse tissues by (^{14}C)-methyl methanesulphonate (dose: 1.2 mM/Kg body weight) - expressed as molar percent of total bases.

(Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

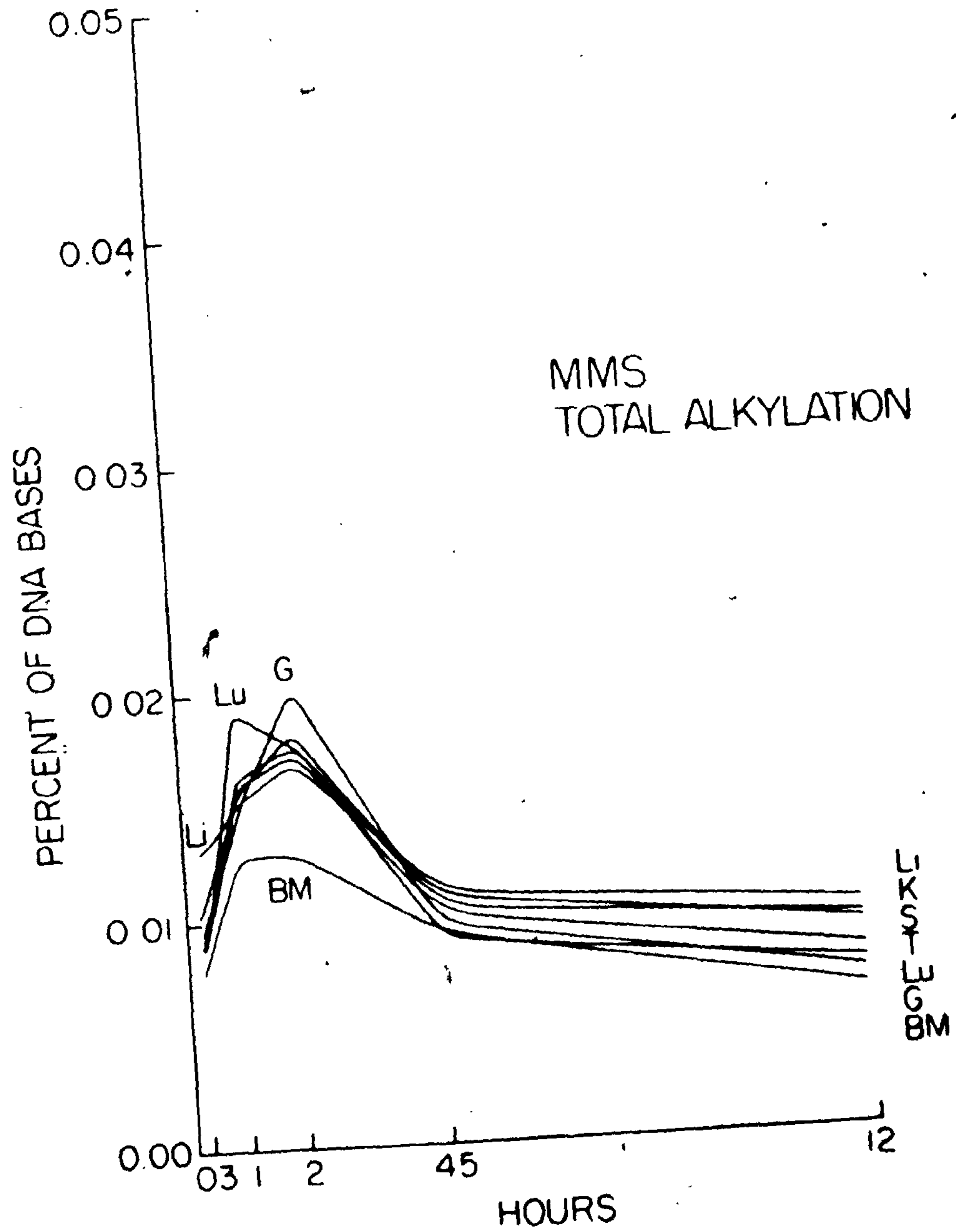


FIG. 16

Fig. 17. Molar percent of guanine methylated as 7MeG in the DNA of different mouse tissues following treatment with (^{14}C)-methyl methanesulphonate (dose: 1.2 mM/Kg body weight).

(Li = Liver; G = Gut; K = Kidney; Lu = Lung; T - Thymus; S = Spleen; BM = Bone marrow)

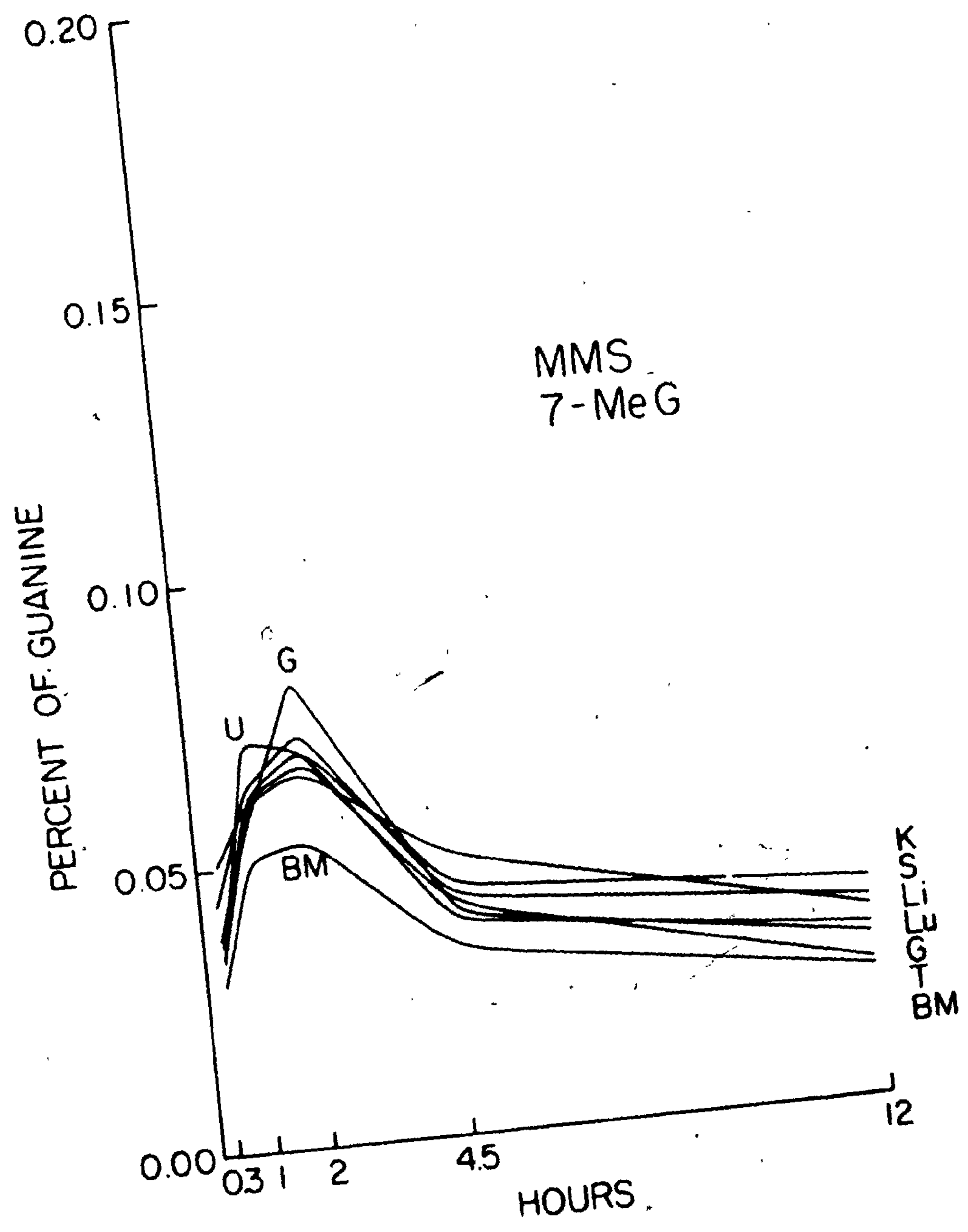


FIG. 17

Fig. 18. Molar percent of adenine methylated as 3MeA in the DNA of different mouse tissues following treatment with (^{14}C)-methyl methanesulphonate (dose: 1.2 mM/Kg body weight). (Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

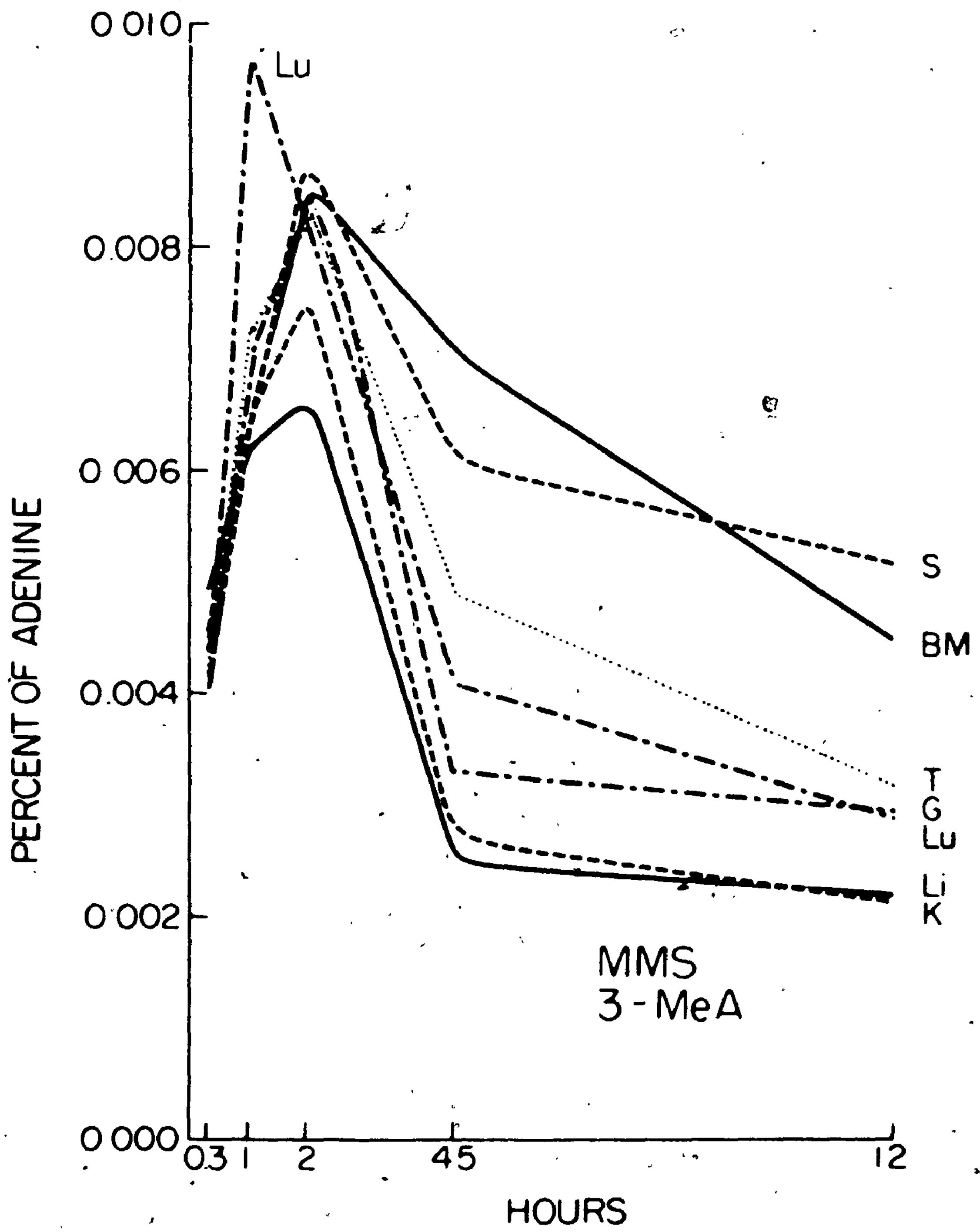


Fig.18

Fig. 19. Molar percent of guanine methylated as O^6-MeG in the DNA of different mouse tissues following treatment with (^{14}C)-methyl methanesulphonate (dose: 1.2 mM/Kg body weight). (Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

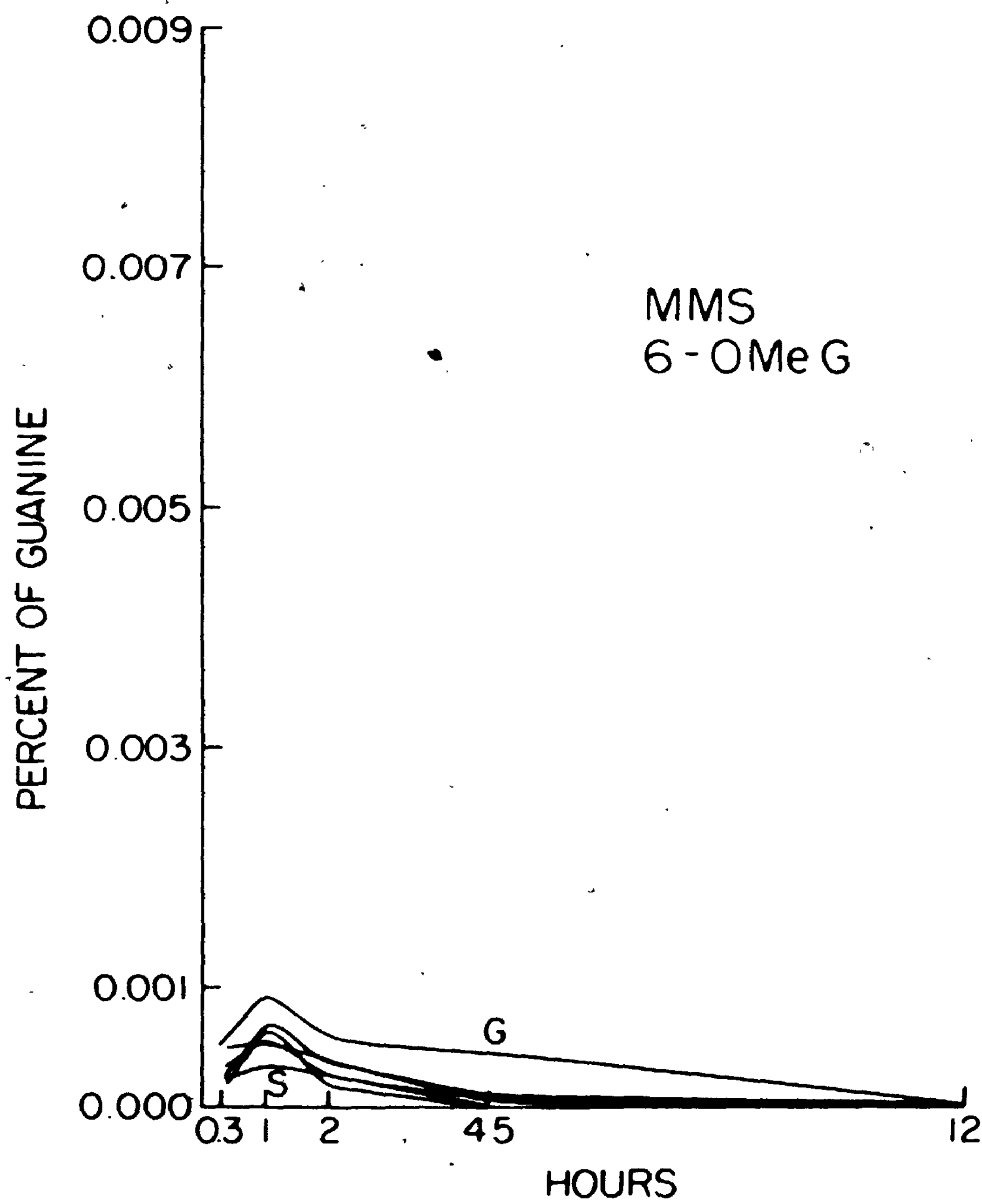


Fig. 19

Fig. 20. Proportion of 7MeG in the DNA of different mouse tissues expressed as percent of total radioactivity due to methylated purines following treatment with (^{14}C)-methyl methanesulphonate (dose: 1.2 mM/Kg body weight). (Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow).

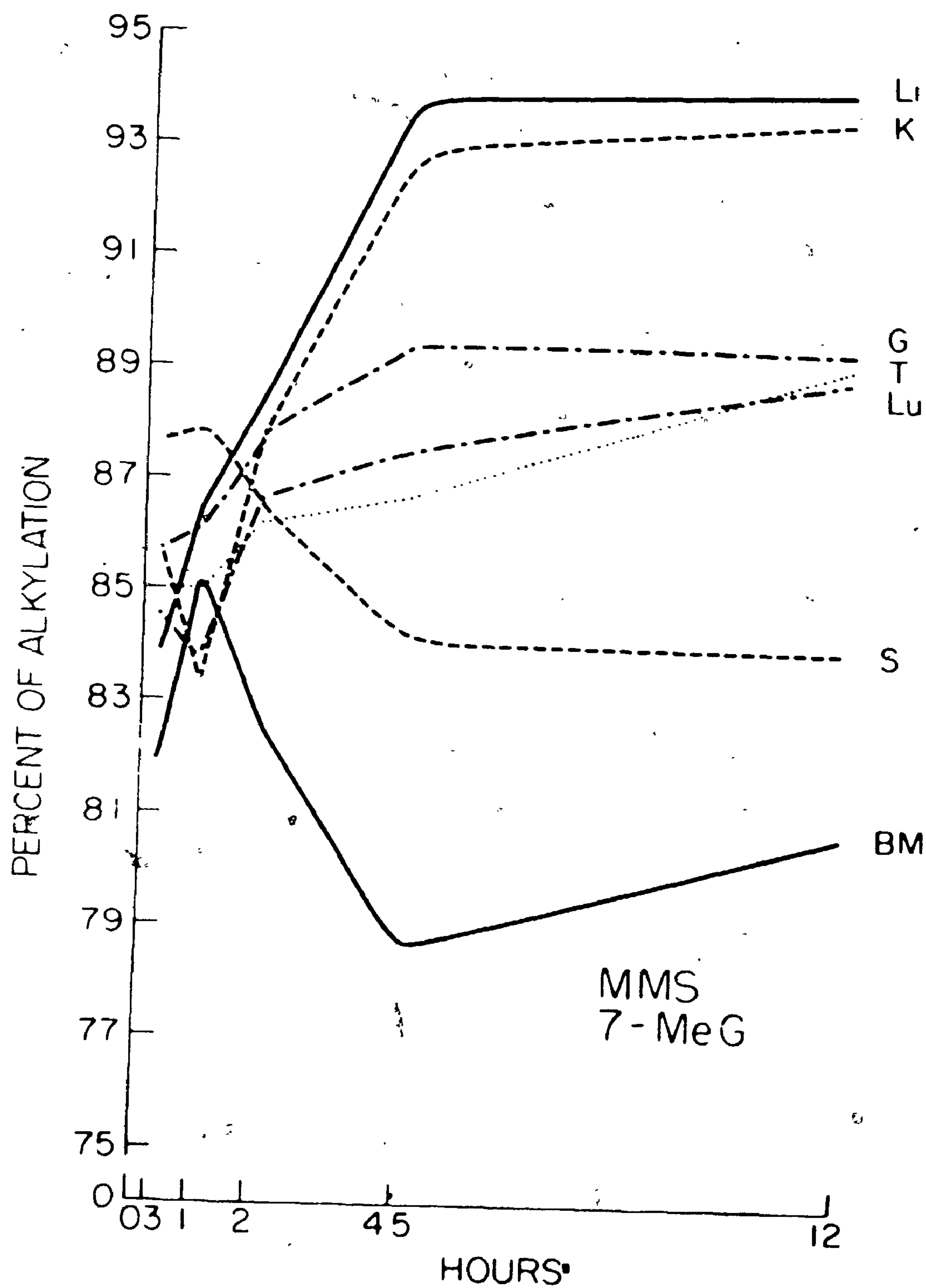


FIG. 20

Fig. 21. Proportion of 3MeA in the DNA of different mouse tissues expressed as percent of total radioactivity due to methylated purines following treatment with (^{14}C)-methyl methanesulphonate (dose: 1.2 mM/Kg body weight).
(Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

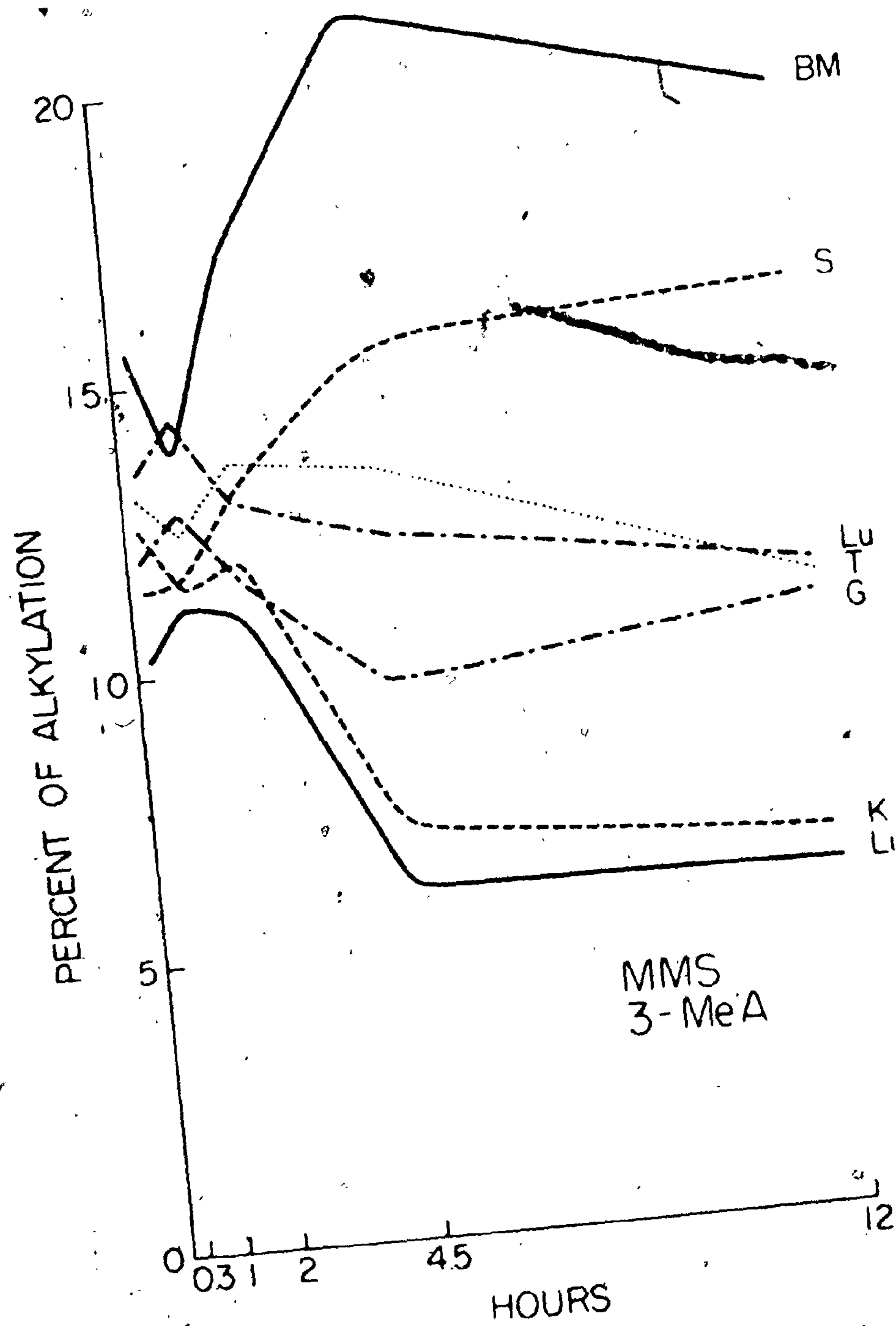


FIG.21

Fig. 22. Metabolic incorporation of labelled carbon (^{14}C) into guanine of DNA in different mouse tissues following treatment with (^{14}C)-methyl methanesulphonate (dose: 0.6 mM/Kg body weight).
(Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow).

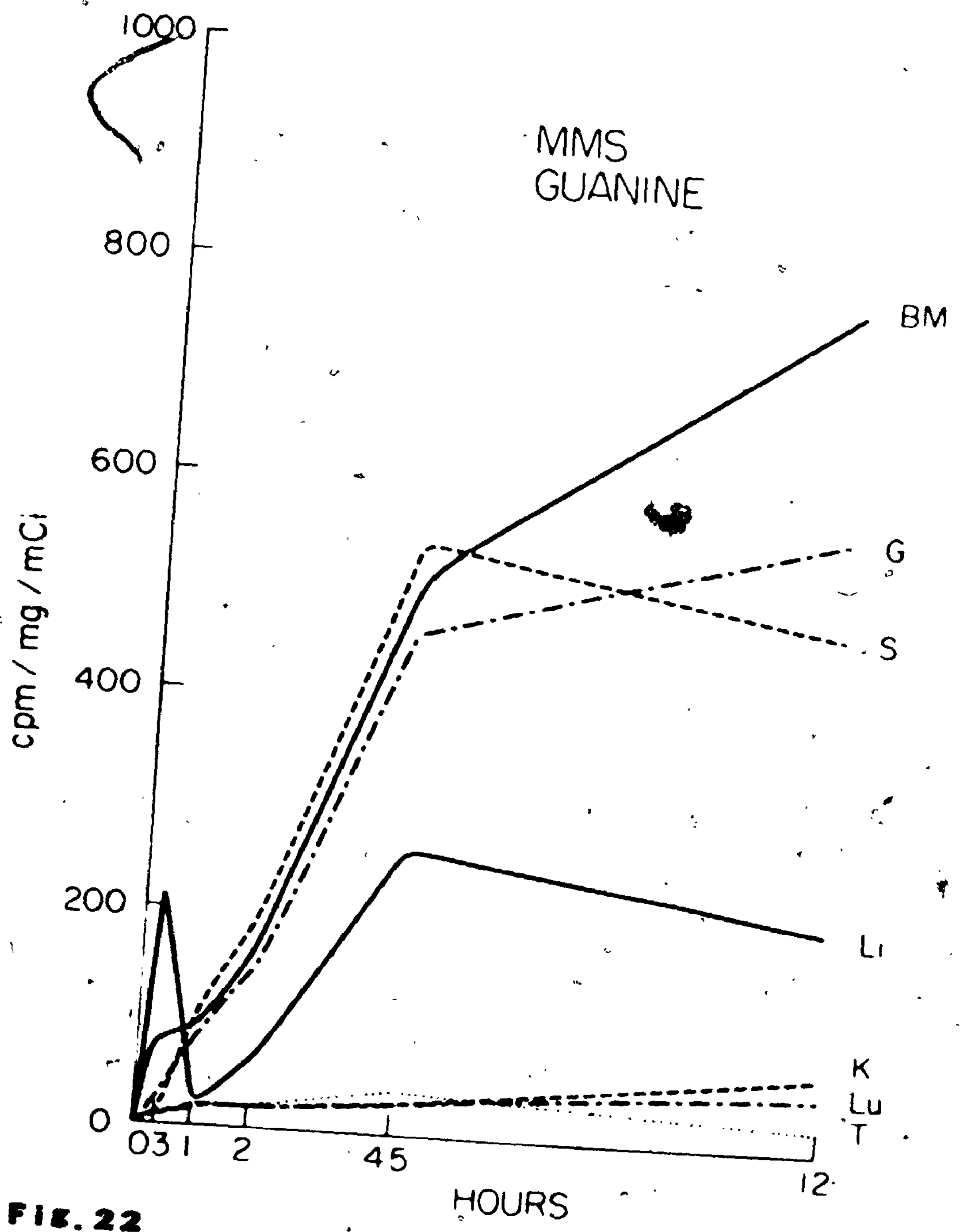


FIG. 22

Fig. 23. Metabolic incorporation of labelled carbon (^{14}C) into adenine of DNA in different mouse tissues following treatment with (^{14}C)-methyl methanesulphonate (dose: 1.2 mM/Kg body weight).

(Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

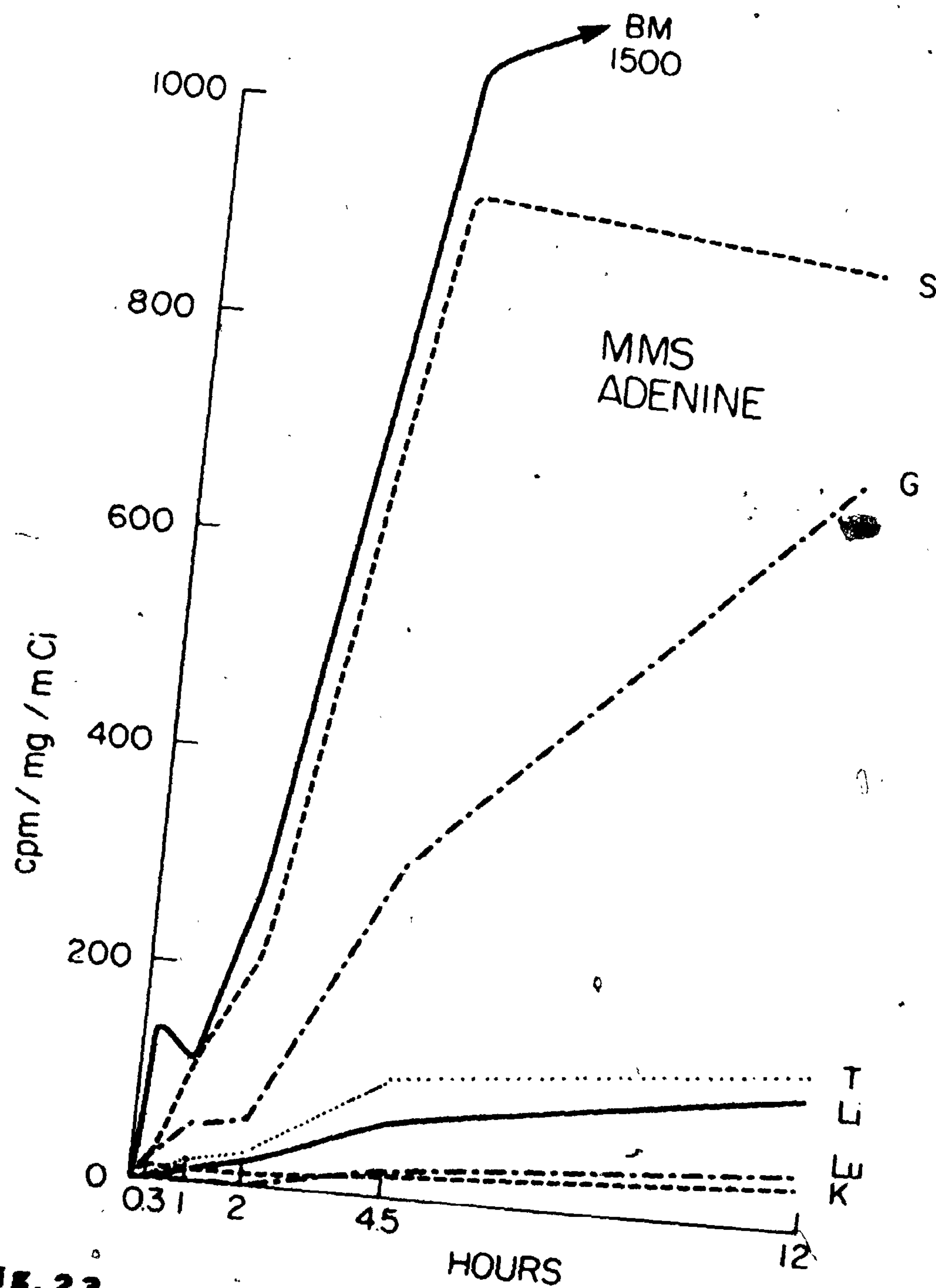


FIG. 23

Fig. 24. Metabolically labelled guanine/adenine ratio in the DNA of different mouse tissues following treatment with (^{14}C)-methyl methane sulphonate (dose: 1.2 mM/Kg body weight) (after normalization).

(Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

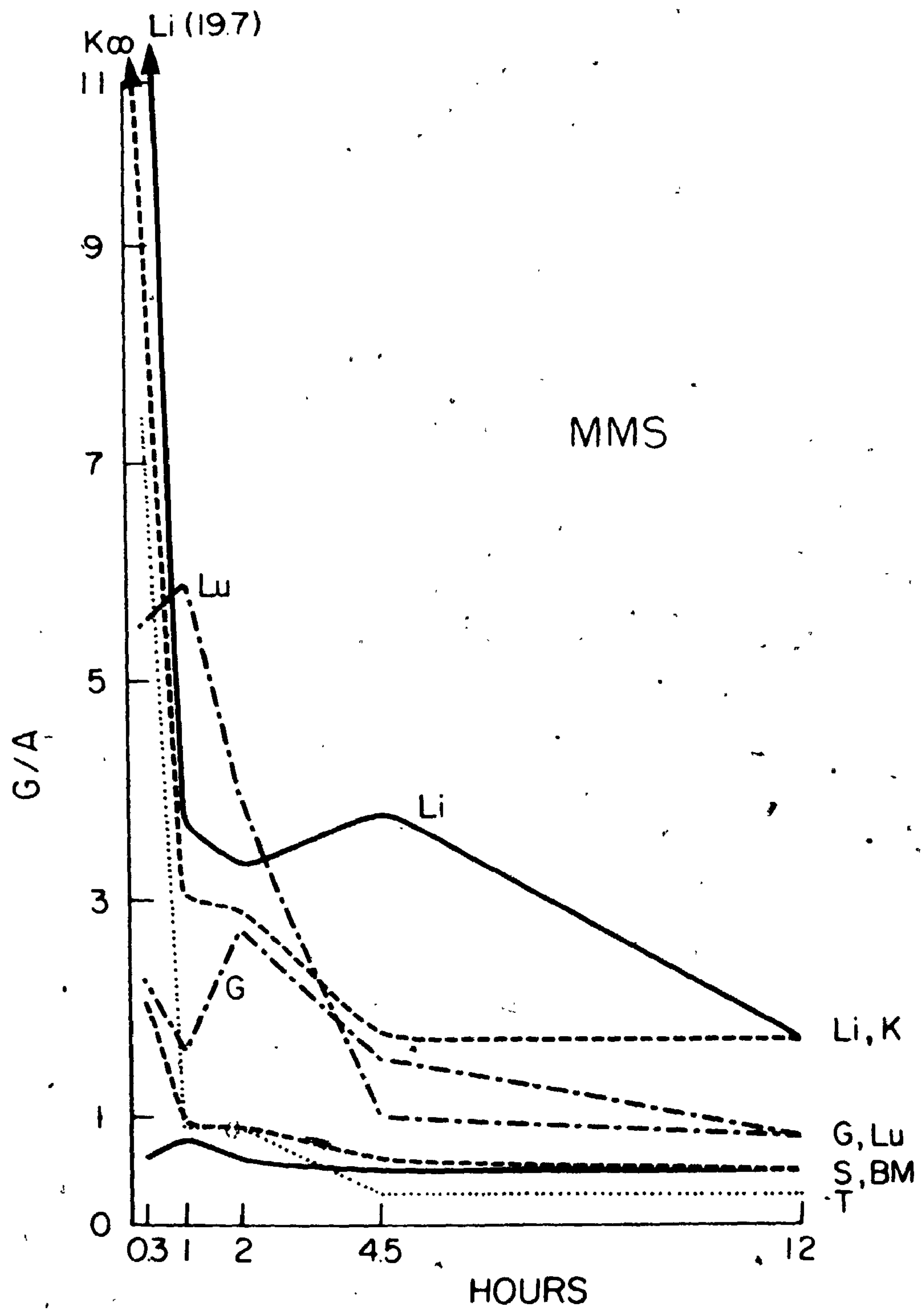


FIG.24

In summary, the following observations were made:

1. In terms of the methylation products studied, there were marked quantitative differences between MNUA and MMS.
2. Significantly more 0^6-MeG was generated by MNUA than by MMS in the DNA of all tissues and this was true at all time points.
3. More 7MeG and 0^6-MeG were generated by MNUA in the DNA of gut, liver and kidney than by MMS; the tissues that are known to develop tumours showed lesser amounts of these two products. No such differences were noted following treatment with MMS.
4. MMS, and possibly with MNUA, 3MeA was retained somewhat longer in tissues other than liver and kidney, particularly bone marrow and spleen.
5. With the exception of bone marrow and spleen, the apparent rates of loss of the reaction products from DNA of all tissues were in the order 3MeA $>0^6$-MeG $>$ 7MeG.

IV. DISCUSSION

The present study was conducted with two alkylating carcinogens, methylnitrosourea (MNUA) and methyl methanesulphonate (MMS). There were three main objectives: to determine the biological effects of MMS at toxicity levels comparable to those of MNUA; to detect, identify and measure the differences, if any, between MNUA and MMS in their reactions with cellular DNA; and to correlate the observations made as above with the genesis of thymic lymphomas in Swiss mice.

* * *

Earlier studies in this laboratory relating to the induction of tumours in this strain of mice indicated that both single and fractionated doses of MNUA given intraperitoneally to adult mice could induce a high incidence of thymic lymphomas (up to 93%) and pulmonary adenomas (0.70/mouse); it was also noted that the main effect of dose fractionation was the enhancement of tumour incidence, weekly doses being more effective than daily doses (for thymic lymphomas - 93% and 75% respectively) (Joshi and Frei, 1970).

In the present work, MMS was tested under the same schedules and was found incapable of inducing tumours of any kind. The results (no thymic lymphoma and only 0.03 pulmonary adenoma/mouse) (see Results in Chapter III - Section A for details) clearly suggest that MMS is a non-carcinogen in this experimental system. Since the development of tumours must be a manifestation of some initial event or events, it is likely that MMS cannot fulfil the necessary conditions needed for neoplasia in this test system.

It was suggested by Frei (1970) that bone marrow and thymus degeneration followed by regeneration might be a step in the genesis of MNUA-induced thymic lymphomas in this strain of mice. The present results (see Results in Chapter III Section B for details) are consistent with such a view. In these experiments MMS failed to cause bone marrow degeneration ($p > 0.01$) although it did cause thymic weight loss ($p < 0.01$); evidences for the same failure were obtained with EMS and MNNG ($p > 0.01$), agents incapable of inducing thymic lymphomas (Frei, 1971a; Frei and Joshi, 1973), whereas ENUA, an inducer like MNUA (Frei, 1971a) caused both bone marrow and thymus changes ($p < 0.01$). It thus appears likely that bone marrow degeneration followed by regeneration is an obligatory step in thymoma induction, if it is accompanied by thymic degeneration and regeneration, though, of course, other factors such as alkylation of DNA may be required as well.

This interpretation is in agreement with the observations of Block (1966) using various manipulations of the induction of thymic lymphomas in mice by x-rays (Table 25). Inspection of his data strongly suggests that the development of thymic lymphoma depended on the co-existence of a thymic and an extra-thymic factor; they were degeneration followed by regeneration of thymus and bone marrow respectively.

* * *

The second objective of the present work concerned the employment of sensitive methods not only for the detection and determination of the various methylation products in DNA but also for the clear separation of metabolically labelled bases from them particularly in tissues with high cell turnover. For this purpose, a new descending paper chromato-

TABLE 25

EFFECT OF VARIOUS MANIPULATIONS IN THE INDUCTION OF THYMIC LYMPHOMAS IN MICE BY X-RAYS[a]

Treatment	Thymus degn.	Thymus regn.	Myeloid degn.	Myeloid regn.	Thymoma
Total body irradiation	+	+	+	+	+
Local irradiation over the thymus	+	+	-	-	-
Total body irradiation with shielded thymus	-	-	+	+	-
Total body irradiation + injection of bone marrow cells	+	+[b]	+	+[b]	-
Total body irradiation + transplanted thymus	+	+	+	+	+
Total body irradiation + testosterone	+	-	+	+	-

a Prepared from the data of Block (1966)

b It is probable that this rapid regeneration was due to injected cells which were not irradiated and thus not capable of neoplasia. They displaced the host's own cells.

graphic technique was devised and employed in the present work.

The decision to attempt to improve the descending paper chromatography procedures, starting from those used previously (Frei, 1971b), rather than to use more recently developed column chromatography procedure of Lawley and Shah (1972a) had to be taken because of the difficulty and the cost of obtaining adequate quantities of sufficiently labelled DNA from small organs such as the bone marrow required for the two types of column chromatography needed for the required separations. Satisfactory improvements were achieved by modifications in the solvent system to give the maximum possible separation of the compounds sought after, by the lengthening of the paper strip, by the use of double exposure to the same solvent and by partial re-chromatography in a second solvent. During re-chromatography, elution from the first chromatogram and reapplication to a second strip were avoided; instead, the appropriate portion of the original chromatogram was fixed in the reverse order to another strip for development. This procedure permitted the needed separation of the minor product 0^6-MeG from contaminants in short runs without any loss of radioactivity that might occur during elution (see sample chromatograms - Figs. 4 & 5).

Modifications were also made in the commonly used mild acid hydrolysis procedure. Since there is known to be very little methylation of pyrimidines (Lawley and Thatcher, 1970; O'Connor et al., 1973), the main purpose of the modified design was to release all the methylated purines with as little destruction as possible and this was achieved by hydrolysis of DNA in 0.1 N HCl at 100°C for 30 minutes.

Validity of the present data:

The present analyses demonstrate both identified and unidentified radioactivity. It is known that alkylating agents react with DNA bases at various sites but whether they esterify phosphates still remains debatable. Two reports are at present available that provide compelling evidence in favour of phosphate alkylation mostly in the form of triesters; the reports further show higher triester formation with SN_1 agents than with SN_2 agents (Bannon and Verly, 1972; Lawley, 1973).

The unidentified radioactivity was always detected in the area from the "origin" to the first identified product, i.e., guanine, in the chromatogram (Fig. 4). The proportion varied from 10 to 50% but averaged about 30% in the MNUA series and 20% in the MMS series initially, rising to about 40% at later times (Fig. 25).

The nature of this material is unknown. It could be, judging from the work of others (Bannon and Verly, 1972; Lawley, 1973), phosphate methylation, especially with MNUA, and this would account for the higher amount of unidentified material with MNUA than with MMS in the first two hours. At later times, a contribution by metabolic incorporation of labelled carbon into pyrimidine bases is to be expected (O'Connor, et al., 1973). Contamination by other methylated or metabolically labelled biological macromolecules, such as RNA, protein, and glycogen could conceivably account for this unidentified radioactivity and for its variation from sample to sample depending on the effectiveness of the DNA isolation procedure. Such contamination ought not to be significant because in the isolation procedure used, measures were taken with all the tissues at all

Fig. 25. Mice treated with either (^{14}C)-methylnitrosourea (dose: 0.6 mM/Kg body weight) or (^{14}C)-methyl methanesulphonate (dose: 1.2 mM/Kg body weight) - DNA isolated from different organs at various intervals, hydrolysed and chromatographed. The unidentified radioactivity is expressed as percent of total radioactivity.
(Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

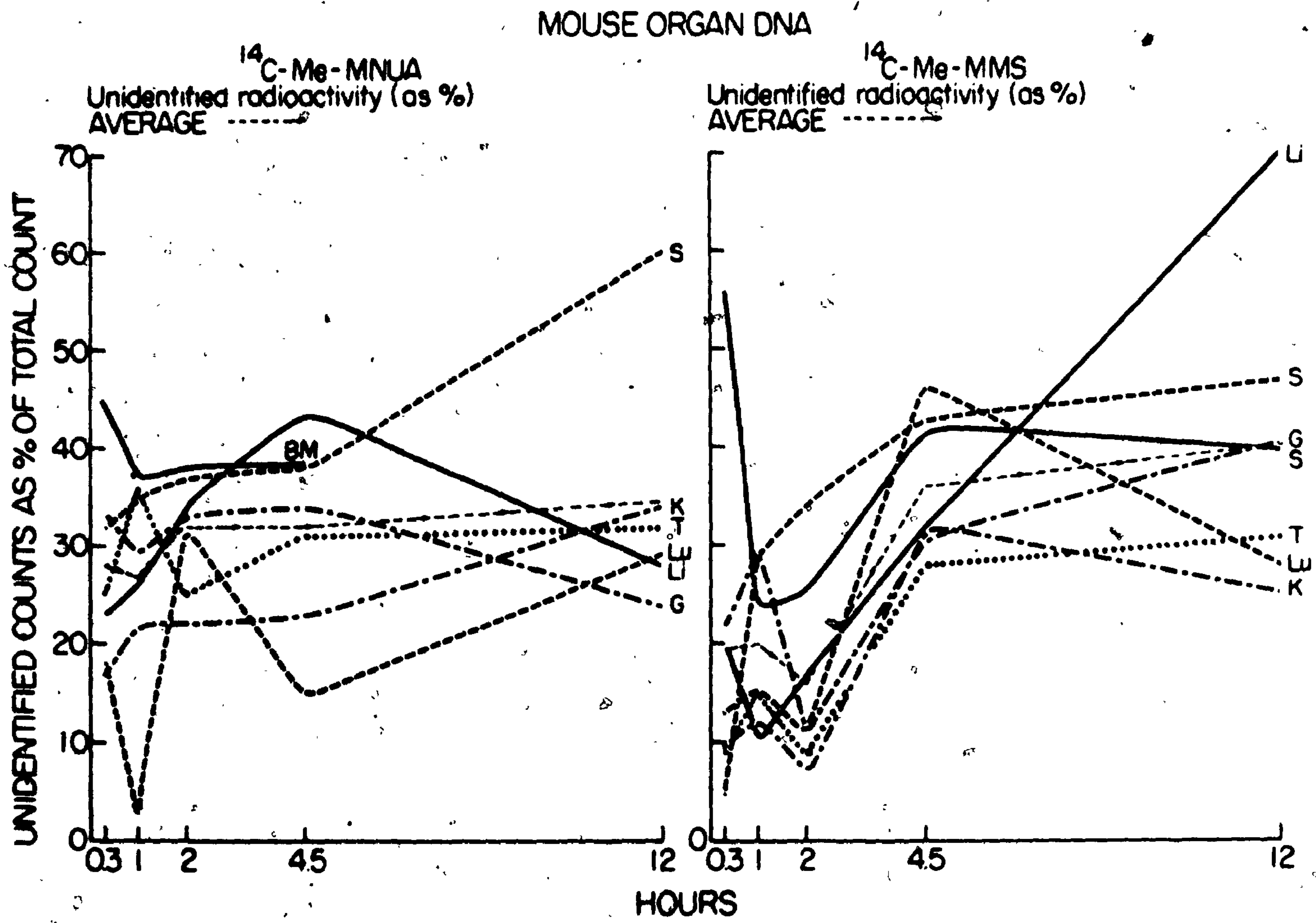


FIG. 25

time points to obtain samples of DNA free from the known common contaminants (e.g., RNase treatment and ethoxyethanol precipitation to eliminate RNA; double phenol extraction to eliminate proteins; and high speed centrifugation to eliminate glycogen). It is not possible, however, to say with certainty that the last trace of impurity was eliminated.

The unidentified material does not contain unreleased DNA purines (methylated or not) because these are known to be liberated quantitatively by the hydrolysis procedure used (see Table 26 showing a comparison of analyses of the same samples of DNA by Dr. P. D. Lawley and the author).

The identified radioactivity consists of metabolically labelled as well as methylated purines. It shows that there is measurable metabolic incorporation of the ^{14}C-labelled methyl group of MNUA or MMS into guanine and adenine; the extent of this incorporation increases with time; as expected, there is a marked tissue variation with the greatest incorporation in tissues that contain the highest proportion of cycling cells. In some samples of DNA, 90% of all the radioactivity is accounted for by the metabolically labelled purines (Fig. 26). A feature of major importance in this work is the clear separation of methylated purines from metabolically labelled purines. A failure to achieve a full separation would result in over estimation of the extent of DNA methylation (Frei, 1971b).

The time-course pattern of alkylation with MMS reveals that the maximal methylation is attained at about 2 hours (Fig. 16). Similar studies with MNUA do not permit to state with certainty the timing of peak methylation because of a large standard deviation in analytical results between tissues and between products. However, it appeared to occur some

TABLE 26

GUT DNA ISOLATED 4.5 HOURS AFTER MNUA AND MMS

Comparison of analyses done by Dr. P. D. Lawley and the author

(expressed as percent of all dpm)

	MMS		MNUA	
	Lawley	Maitra	Lawley	Maitra
Unidentified activity	19	31	27	34
Guanine + Adenine	57	47	30	33
Total (not alkylated bases)	76	78	57	67
7-methylguanine	19	20	36	29
3-methyladenine	1.4	2.2	0.8	1.6
6-methoxyguanine	0.08*	0.2*	4.7	1.9

* Not significant

Dose: MNUA - 0.6 mmole/Kg body weight intraperitoneally

MMS - 1.2 mmole/Kg body weight intraperitoneally

Fig. 26. Mice treated with either (^{14}C)-methylnitrosourea (dose: 0.6 mM/Kg body weight) or (^{14}C)-methyl methanesulphonate (dose: 1.2 mM/Kg body weight) - DNA isolated from different organs at various intervals, hydrolysed and chromatographed. Radioactivity due to labelled guanine + adenine is expressed as percent of total known radioactivity.

(Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

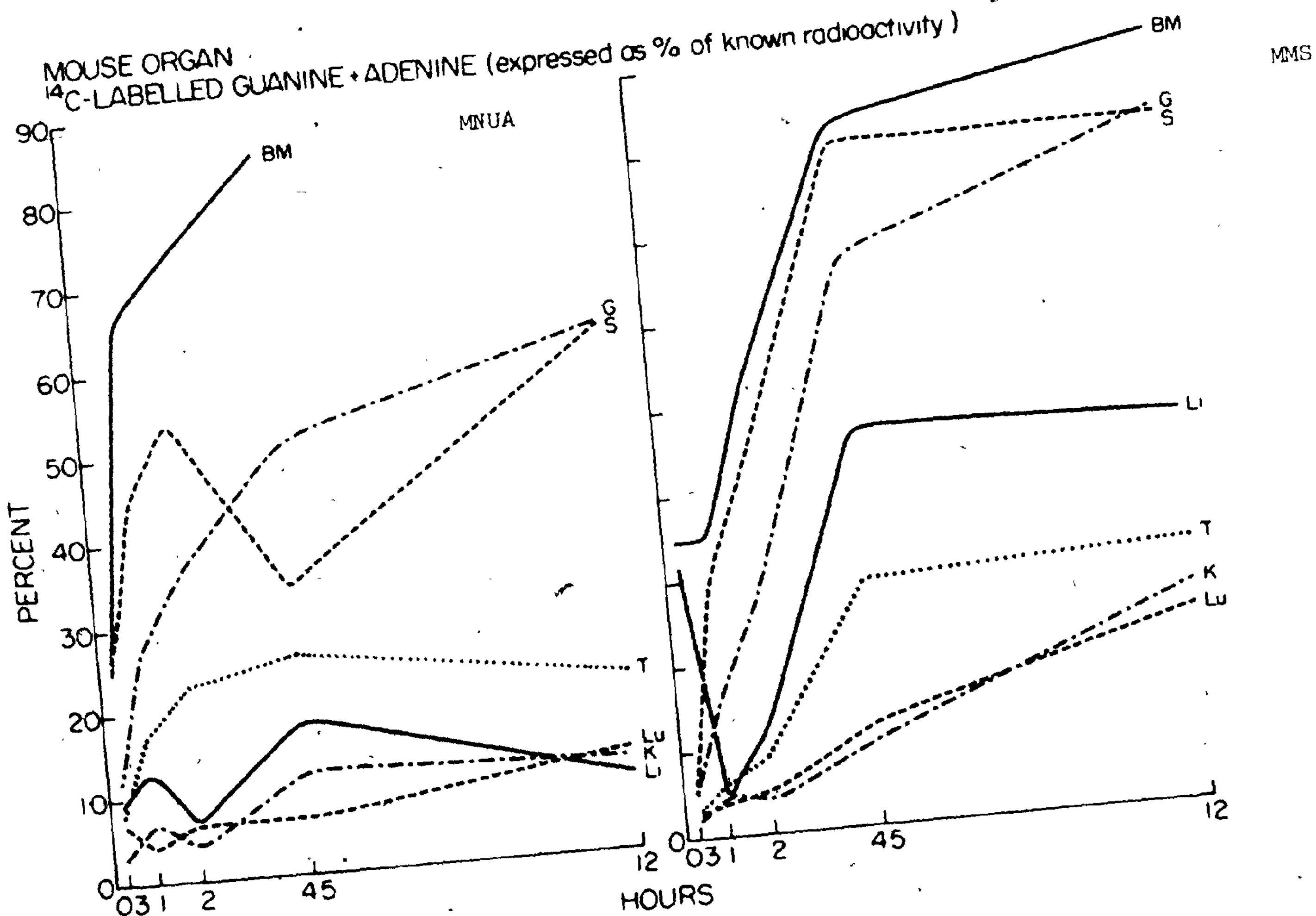


FIG. 26

time around 2 to 4.5 hours.

Of the several factors that may influence the extent of alkylation either in vitro or in vivo, the one which is probably the most important is the half life of the agent concerned. The half lives of MNUA and MMS in different mouse tissues are not known but it may be expected that they would not be longer than what they are in vitro under physiological conditions. At pH 7.4 and 37°C, MNUA has a half life of about 16 minutes while MMS has a half life of about 5 hours (Thatcher, 1971). Some information about the life time of these two agents in rats is available; given single doses intravenously, the life times of MNUA and MMS have been found to be 15 minutes and 1.5 hours respectively (Swann, 1968). From these data, it may be expected that the half lives of MNUA and MMS in mice are likewise short and the bulk of alkylation would be over by about 30 minutes in case of MNUA and by about 3 hours in case of MMS.

Following treatment with MMS, the observed timing of maximal alkylation (statistically shown in Table 27) conforms satisfactorily with the reported rate of hydrolysis of this agent in vivo and agrees with the observations of O'Connor et al. (1973) that the highest level of alkylation in rat liver DNA was achieved between 2 and 4 hours.

Following treatment with MNUA, the time-course pattern of alkylation appears not to agree with the known rate of hydrolysis of this agent because methylation of DNA seems to continue well beyond 30 minutes. In preliminary experiments of a similar nature done independently by Dr. J. V. Frei with MNUA, an almost identical pattern of alkylation was observed, i.e., maximal alkylation occurred at about 4 hours, despite errors

TABLE 27

Part I - Total alkylation of DNA following MMS expressed as dpm/mg DNA/mCi/mmole of dose with mean, variance and standard deviation.

Tissue	At 0.3 hr	At 1 hr	At 2 hrs	At 4.5 hrs	At 12 hrs
Gut	536	748	1160	554	414
Liver	756	886	954	649	587
Spleen	619	917	1052	631	521
Thymus	506	927	968	589	477
Bone marrow	447	750	809	532	371
Lung	507	1072	1007	544	423
Kidney	531	897	999	603	526
$\overline{X}$	557	885	993	586	474
V	10294	12483	11267	1999	5750
s.d.	101	112	106	45	76

Part II - Comparison of the means

Time	Calculated 't'	Significance ($p<0.01$)
0.3 hr vs 2 hrs	7.86	+
2 hrs vs 12 hrs	10.84	+

in quantitation due to imperfect chromatograhic separation of guanine and 7MeG (Fig. 27). The similarity of these two patterns may be viewed as a confirmation of the present observation. The reason for the discrepancy between the time-course of alkylation and the half life remains unclear at present.

The proportions of alkylated purines are, in general terms, much like those reported in the literature for other DNAs alkylated by the same agents either _in vitro_ or _in vivo_ (Table 28). An analysis of some of the samples by Dr. P. D. Lawley shows good agreement with the present results with one exception (Table 29). The proportion of 0^6-MeG in the system used here is about half of that determined by Dr. Lawley. Part of this is accounted for by destruction of this product under the hydrolytic conditions used. However, measurement made on the destruction of pure 0^6-MeG indicated that the results would be lower by only about 25%. The rest of the difference appears a systematic one, i.e., involving all samples about the same way. This conceivably could be due to an RNA contaminant since RNA is reported to contain less 0^6-MeG (Table 30), thus lowering the total proportion of this product. But such a possibility is unlikely. The full explanation of this anomaly is not clear at the moment but excessive destruction beyond that determined by the study of the authentic compound appears likely. The present data for 0^6-MeG should thus be interpreted as positive identification of 0^6-MeG (or absence) but the validity of the observed changes in its level with time yet remains to be established.

Fig. 27. Molar percent of guanine methylated as 7MeG in the mouse liver DNA following treatment with (^{14}C)-methylnitrosourea (dose: 0.6 mM/Kg body weight)(preliminary data of Dr. J. V. Frei).

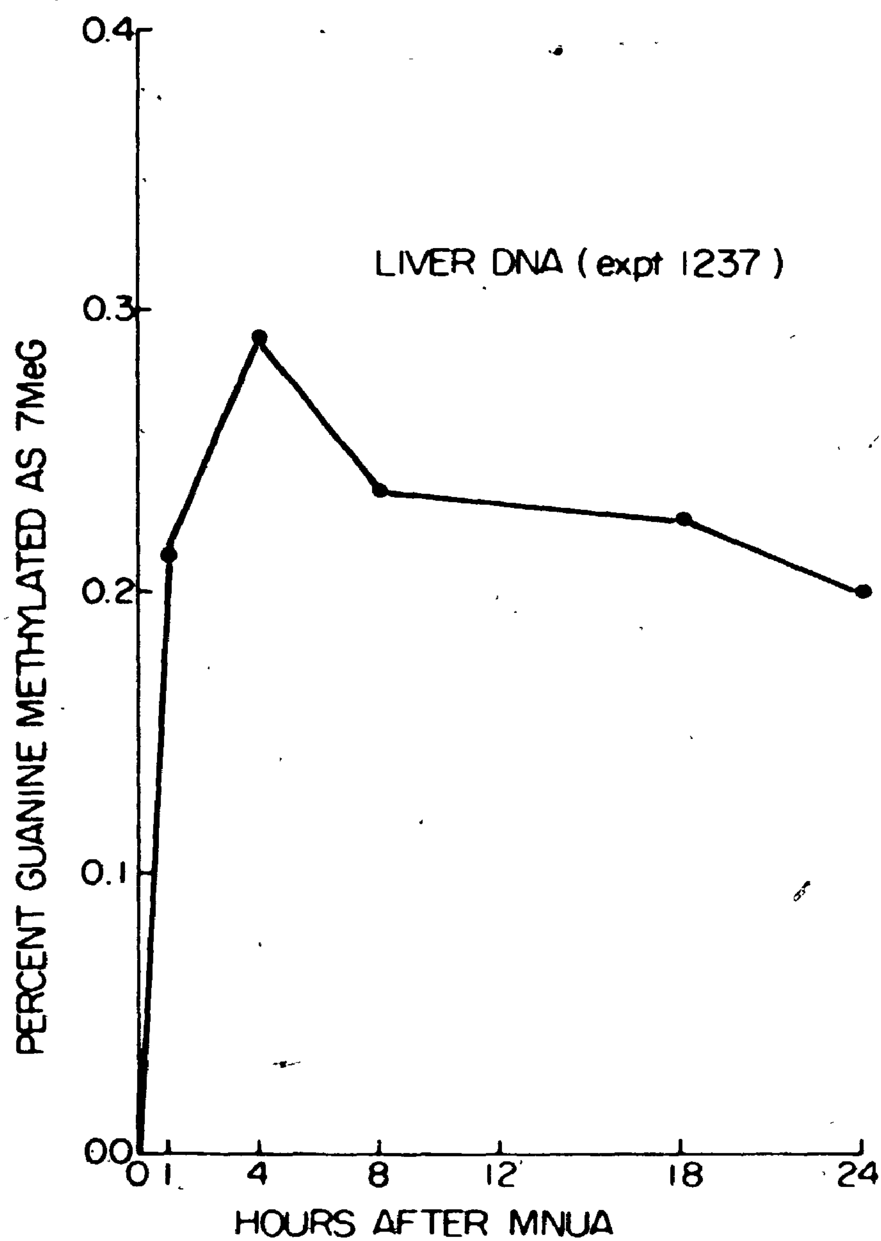


FIG. 27

TABLE 28

Methylated purines in DNA expressed as percent of total methylated purines

	After MNUA			After MMS	
	in vivo [a] (L cells)	*in vitro* (Salmon sperm DNA) [b]	[c]	*in vivo* [d] (Rat liver)	*in vitro* [e] (Calf thymus DNA)
Treatment	40 min.	1 hr.	1 hr.	4 hrs.	18 hrs.
7-methylguanine	82.3	91.3	75.7	91.3	86.0
3-methyladenine	9.4	0.7	11.2	7.2	10.0
O^6-methylguanine	8.2	8.0	7.3	-	-
1-methyladenine	-	-	1.4	0.6	4.0
3-methylguanine	-	-	1.1	0.9	-

a Data from Thatcher (1971)

b Data from Dr. I.G. Walker (personal communication)

c Data from Lawley and Shah (1973)

d Data from Craddock (1973)

e Data from Lawley and Brookes (1961)

TABLE 29

GUT AND LIVER DNA ISOLATED 4.5 HOURS AFTER MNUA

Comparison of analyses done by Dr. P. D. Lawley and the author

(expressed as percent of all purines)

	Gut		Liver	
	Lawley	Maitra	Lawley	Maitra
7-methylguanine	85	89	82	92
3-methyladenine	2	5	4	4
6-methoxyguanine	11	6	10	3
Other methylated purines	<5		<5	

Dose of MNUA: 0.6 mmole/Kg body weight - intraperitoneally

TABLE 30

METHYLATED BASES IN RNA EXPRESSED AS PERCENT OF TOTAL METHYLATED BASES

	in vitro[a]		in vivo[b]	
	MNUA	DMS	DMN	MMS
1-methyladenine	3	13	1.6	10
3-methyladenine	0.7	1	1	3
7-methyladenine	2	2	0.6	1.9
3-methylcytosine	1	12	2	8
7-methylguanine	80	60	85	75
6-methoxyguanine	3	trace	4	trace

a Data from Lawley and Shah (1972)

b Data from O'Connor et al. (1972)

Evaluation of the results:

The study of the alkylation products due to MMS shows a near-identity of the results in all tissues. If, therefore, the tissues are considered as multiple samples, a statistical evaluation becomes possible. In order to do this all the data were normalized as dpm/mg DNA/mCi/mmole of dose given (Table 31) and the means and s.d's plotted (Fig. 28). The MNUA data were treated the same way for comparative purposes (Table 32, Fig. 28).

Using this approach, the following definitive observations can be made:

a) The variance ratios between the corresponding MNUA and MMS data for 7MeG show that the MNUA s.d's are significantly larger than the MMS ones ($p < 0.01$) indicating that the tissue differences in the level of 7MeG seen with MNUA are significant compared to MMS (Table 33 - Part I); the level of 7MeG with MNUA is in the order liver > gut (large and small) > kidney > lung > thymus > spleen > bone marrow. This is in good agreement with the observations of Kleihues and Magee (1973) that the level of 7MeG following MNUA at 2 hours in rats was in the order liver > brain > ileum. Tissue differences of similar nature are also possibly true with respect to the level of O^6-MeG (Fig. 28) but due to the facts already discussed such a conclusion cannot be drawn with certainty.

b) The 3MeA variance ratios between the corresponding MNUA and MMS data, on the other hand, except at 0.3 hours (20 minutes) are much smaller and several are not significant (Table 33 - Part I). Therefore, the tissue differences in the level of 3MeA may be significantly less than those in 7MeG .

TABLE 31

Methylation products of DNA following treatment with MMS (dose: 1.2 mM/Kg body weight), with mean, variance and standard deviation (expressed as dpm/mg DNA/mCi/mmole dose)

Tissue	At 0.3 hr			At 1.0 hr		
	7MeG	3MeA	0^6MeG	7MeG	3MeA	0^6MeG
Gut	459	64.3	5.9	726	111	12.2
Liver	634	77.9	6.8	766	99	7.1
Spleen	543	71.2	3.1	806	106	4.6
Thymus	430	66.3	3.0	788	115	9.2
Bone marrow	366	69.7	4.0	639	103	7.5
Lung	429	68.4	2.5	896	154	8.6
Kidney	455	66.9	3.2	772	103	7.2
$\overline{X}$	474	69.2	4.1	770	113	8.1
V	7762	19.7	2.7	6152	356	5.5
s.d.	88	4.4	1.6	78	18.9	2.3

TABLE 31

Methylation products of DNA following treatment with MMS (dose: 1.2 mM/Kg body weight), with mean, variance and standard deviation (expressed as dpm/mg DNA/mCi/mmole dose)

At 2.0 hrs			At 4.5 hrs			At 12 hrs		
7MeG	3MeA	O^6MeG	7MeG	3MeA	O^6MeG	7MeG	3MeA	O^6MeG
1018	135	7.0	495	53.2	5.5	370	43.9	-
844	105	4.8	609	39.6	-	553	35.3	-
910	139	4.2	530	97.7	2.5	438	83.3	-
834	132	2.0	511	78.4	-	425	52.0	-
668	138	3.2	419	112.4	1.1	300	71.6	-
872	130	5.0	476	66.4	1.6	376	47.0	-
876	119	4.0	560	42.8	-	492	34.2	-
860	128	4.3	514	70.1		422	52.5	
10943	154	2.5	3706	766		7005	341	
105	12.4	1.6	60.9	27.7		83.7	18.5	

Fig. 28. Mice treated with either (^{14}C)-methylnitrosourea (dose: 0.6 mM/Kg body weight) or (^{14}C)-methyl methanesulphonate (dose: 1.2 mM/Kg body weight) - DNA isolated from different organs at various intervals, hydrolysed and chromatographed. Shows mean values of the individual products ± s.d. (after normalization).

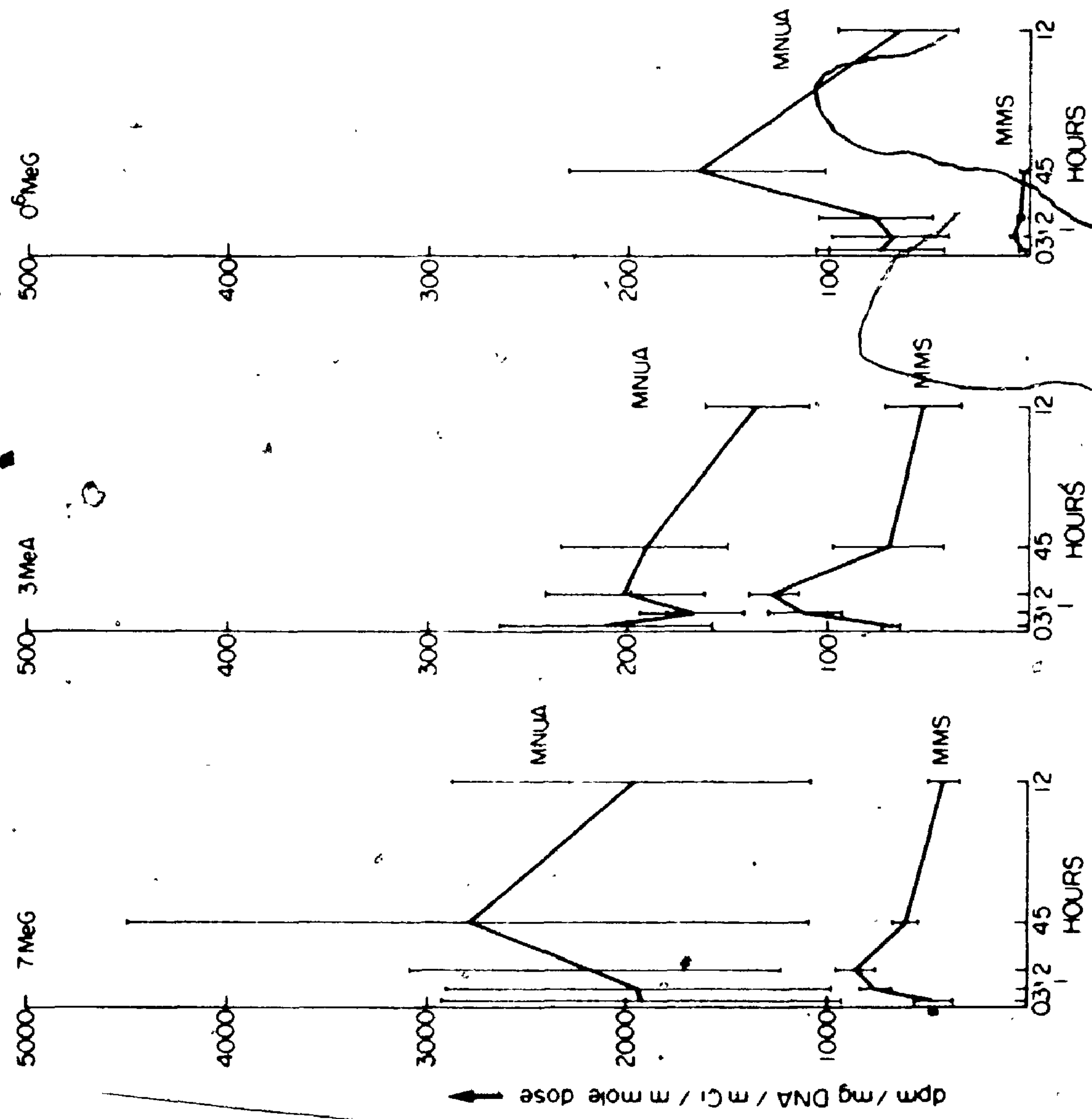


FIG. 28

TABLE 32

Methylation products of DNA following treatment with MNUA (dose: 0.6 mM/Kg body weight), with mean, variance and standard deviation (expressed as dpm/mg DNA/mCi/mmole dose)

Tissue	At 0.3 hr			At 1.0 hr		
	7MeG	3MeA	O^6MeG	7MeG	3MeA	O^6MeG
Gut	2344	200	52	2795	175	98
Liver	3966	274	141	3562	209	119
Spleen	1356	215	72	1338	149	49
Thymus	1246	152	52	878	142	40
Bone marrow	1217	209	91	1124	139	69
Lung	1406	145	63	1927	187	63
Kidney	1940	283	57	1999	172	45
$\overline{X}$	1925	211	75	1946	168	69
V	982816	2855	1024	920233	664	866
s.d.	991	53.4	32	959	25.8	29.4

TABLE 32

Methylation products of DNA following treatment with MNUA (dose: 0.6 mM/Kg body weight), with mean, variance and standard deviation (expressed as dpm/mg DNA/mCi/mmole dose)

At 2.0 hrs			At 4.5 hrs			At 12 hrs		
7MeG	3MeA	0^6MeG	7MeG	3MeA	0^6MeG	7MeG	3MeA	0^6MeG
2970	196	95	3300	189	215	2286	165	92
3704	269	124	6329	276	235	3612	153	113
1755	214	67	1721	166	110	1271	158	40
1304	147	36	1833	201	83	1493	123	43
1375	176	96	1339	201	147	-	-	-
1492	183	59	2241	166	128	1280	100	49
2555	236	63	2816	145	242	1917	123	64
2165	203	77	2797	192	166	1976	137	67
859182	1647	866	2875061	1798	4128	798161	648	876
927	40.6	29.4	1696	42.4	64.2	893	25.5	29.6

TABLE 33

Part I: VARIANCE RATIOS MNUA/MMS (Mean values)

	7MeG		3MeA	
Time	Ratio	Significance ($p<0.01$)	Ratio	Significance ($p<0.01$)
0.3 hr.	127	+	145	+
1 hr.	150	+	1.81	-
2 hrs.	79	+	10.69	+
4.5 hrs.	775	+	2.35	-
12 hrs	114	+	1.90	-

Part II: DIFFERENCE OF MEANS - MNUA vs. MMS

	7MeG		3MeA	
Time	Calculated 't'	Significance ($p<0.01$)	Calculated 't'	Significance ($p<0.01$)
0.3 hr.	3.86	+	7.48	+
1 hr.	3.23	+	4.56	+
2 hrs.	3.70	+	4.68	+
4.5 hrs	3.56	+	6.37	+
12 hrs.	4.61	+	6.93	+

c) Comparison of the means between the first, maximal and last values for the MNUA data are not statistically significant for 7MeG, indicating that the rises and falls and the timing of peak level cannot be deduced from the data as already noted. 3MeA and 0^6-MeG, on the other hand, show significant declines from peak levels (Table 34). While the average level of 3MeA is seen to go down to about 67% of the average peak level over the period from 2 to 12 hours, the actual extent of decline of 0^6-MeG over the period from 4.5 to 12 hours cannot be ascertained due to possibility of unknown losses during hydrolysis.

d) Comparison of the means between the first, maximal and last values for the MMS data show significant rise and fall in the levels of 7MeG and 3MeA (Table 34). The average level of 7MeG is seen to go down to about 50% while the level of 3MeA goes down to about 40% of the average peak levels over the period from 2 to 12 hours.

e) On a molar basis, as shown by significant differences in means (Table 33 - Part II), MMS is less effective in alkylation at N-7 position of guanine and N-3 position of adenine, than MNUA.

f) MNUA generates 0^6-MeG whereas MMS produces very little, if any, of it (Fig. 28).

The extent of DNA base alkylation in different tissues at any particular time is probably a reflection of the total effect of two opposing sets of conditions acting simultaneously or in sequence.

Those included into the list of conditions enhancing the observed alkylation are:

i) Availability of the alkylating species. This would depend, as already discussed, primarily on the rate of hydrolysis of the agent and its penetration to the requisite site in the tissue. However,

TABLE 34

DIFFERENCE OF MEANS - PEAKS vs. 0.3 HR. OR 12 HRS.
OR 1ST AND LAST VALUES.

Product	MNUA		
	Time	Calculated 't'	Significance ($p<0.01$)
7-methylguanine	4.5 hrs vs 0.3 hr	1.17	-
	4.5 hrs vs 12 hrs	1.06	-
3-methyladenine	0.3 hr vs 12 hrs	3.09	+
	2 hrs vs 12 hrs	3.44	+
0^6-methylguanine	4.5 hrs vs 0.3 hr	3.36	+
	4.5 hrs vs 12 hrs	3.14	+

Product	MMS		
7-methylguanine	2 hrs vs 0.3 hr	7.47	+
	2 hrs vs 12 hrs	8.66	+
3-methyladenine	2 hrs vs 0.3 hr	11.8	+
	2 hrs vs 12 hrs	8.99	+

Alexander et al. (1961) and Lett et al. (1962) produced evidence for a slow transalkylation reaction under physiological conditions from tri-ester phosphates to DNA bases following alkylation by monofunctional nitrogen mustard. If such a process does also operate in vivo. it might raise the overall level of base alkylation with time.

ii) Poorly alkylated cells may be eliminated by cell death and thus raise the average level of alkylation. There is no information available to suggest such a situation.

Those included among the conditions decreasing the observed alkylation are:

i) Spontaneous or chemical excision of alkylated bases with consequent single strand breaks in DNA (Strauss et al., 1969). Lawley and Brookes (1963) have demonstrated that at pH 7 and 37°C, 7MeG is removed from DNA with a half life of about 150 hours and 3MeA is removed with a half life of about 25 hours. Methylation of the 0^6-position of guanine in DNA, on the other hand, does not result in chemical lability and the methylated base is stable at 100°C at pH 7 for 20 minutes (Lawley and Thatcher, 1970).

ii) Specific enzyme excision. The available information on the enzymic excision of 7MeG in bacteria (Olson and McCalla, 1969; Lawley and Orr, 1970) or in mammalian cells (Roberts et al., 1971; Margison et al., 1973) is contradictory. However, in bacteria (Lawley and Orr, 1970) and in mammalian cells (O'Connor et al., 1973) the rates of loss of 3MeA and 0^6-MeG suggest that these two products may be excised enzymically.

iii) Cellular necrosis may take place with loss of highly alkylated cells. This would lead to an overall lowering of the level of alkylation of DNA as isolated.

iv) Dilution by the newly synthesized DNA would lead to a lowering of overall level of alkylation, a factor particularly likely to influence the observations on tissues with a high proportion of cycling cells.

Following MMS, 7MeG is lost from the DNA of all tissues faster than can be accounted for by chemical excision (estimated half life is about 10 hours). Since the literature does not definitely implicate active excision process for this product, it is not possible at present to decide whether the observed decline is due to enzymic excision or due to other factors as noted (i.e. dilution etc.). 3MeA, on the other hand, disappears faster, in some tissues with an estimated half life range of 3.5 to 14 hours. Craddock (1973) found the half life of 3MeA in vivo (rat liver DNA) to be about 7 hours. Since the chemical lability of the base does not fully account for this rate of loss, it is likely that 3MeA may be excised enzymically in some tissues.

Further evidence for specific enzymic removal of 3MeA following MMS may be obtained by taking into consideration the ratios of 3MeA/7MeG at different time points. While the affinity of the various positions on the purines for the alkyl group of an agent may vary, the proportion of the alkylated bases cannot change with time unless one or the other is removed preferentially. Such calculations were therefore made (Tables 35 - 37, Fig. 29).

TABLE 35

Ratio of the alkylation products of DNA following MMS

with mean, variance and standard deviation.

(after normalization as dpm/mg DNA/mCi/mmole of dose)

Tissue	At 0.3 hr		At 1 hr		At 2 hrs		At 4.5 hrs		At 12 hrs	
	3MeA/7MeG	O^6MeG/7MeG	3MeA/7MeG	O^6MeG/7MeG	3MeA/7MeG	O^6MeG/7MeG	3MeA/7MeG	O^6MeG/7MeG	3MeA/7MeG	O^6MeG/7MeG
Gut	0.1400	0.0129	0.1529	0.0168	0.1326	0.0069	0.1075	-	0.1186	-
Liver	0.1229	0.0107	0.1292	0.0093	0.1244	0.0057	0.0650	-	0.0638	-
Spleen	0.1311	0.0057	0.1315	0.0057	0.1527	0.0046	0.1843	-	0.1902	-
Thymus	0.1542	0.0070	0.1459	0.0117	0.1582	0.0024	0.1534	-	0.1226	-
Bone marrow	0.1904	0.0109	0.1612	0.0117	0.2066	0.0048	0.2682	-	0.2387	-
Lung	0.1594	0.0058	0.1719	0.0096	0.1490	0.0057	0.1395	-	0.1250	-
Kidney	0.1470	0.0070	0.1334	0.0093	0.1358	0.0046	0.0764	-	0.0695	-
$\overline{X}$	0.1493	0.00857	0.1466	0.01059	0.1513	0.00496	0.1420	-	0.1326	-
V	0.00049	0.00001	0.00027	0.00001	0.00074	0.00000	0.00488	-	0.00394	-
s.d.	0.02212	0.00287	0.01632	0.00340	0.02716	0.00140	0.06985	-	0.06277	-

TABLE 36

Ratio of the alkylation products of DNA following MNUA with mean, variance and standard deviation.

(after normalization as dpm/mg DNA/mCi/mmole of dose)

Tissue	At 0.3 hr		At 1 hr		At 2 hrs		At 4.5 hrs		At 12 hrs	
	3MeA / 7MeG	O^6MeG / 7MeG	3MeA / 7MeG	O^6MeG / 7MeG	3MeA / 7MeG	O^6MeG / 7MeG	3MeA / 7MeG	O^6MeG / 7MeG	3MeA / 7MeG	O^6MeG / 7MeG
Gut	0.0853	0.0222	0.0626	0.0351	0.0660	0.0320	0.0573	0.0651	0.0722	0.0402
Liver	0.0691	0.0356	0.0587	0.0334	0.0726	0.0335	0.0436	0.0371	0.0424	0.0313
Spleen	0.1585	0.0531	0.1114	0.0366	0.1219	0.0382	0.0965	0.0639	0.1243	0.0315
Thymus	0.1220	0.0417	0.1617	0.0456	0.1127	0.0276	0.1097	0.0453	0.0824	0.0288
Bone marrow	0.1717	0.0748	0.1237	0.0614	0.1280	0.0698	0.1501	0.1098	-	-
Lung	0.1031	0.0448	0.0970	0.0327	0.1226	0.0395	0.0741	0.0571	0.0781	0.0383
Kidney	0.1459	0.0294	0.0860	0.0223	0.0924	0.0247	0.0515	0.0860	0.0642	0.0334
$\overline{X}$	0.1222	0.04309	0.10016	0.03819	0.10231	0.03790	0.08326	0.06633	0.07727	0.03392
V	0.00148	0.00030	0.00130	0.00015	0.00064	0.00023	0.00145	0.00061	0.00073	0.00002
s.d	0.03846	0.01728	0.03607	0.01228	0.02537	0.01503	0.03804	0.02472	0.02702	0.00442

TABLE 37

Part I: Variance ratios 3MeA/7MeG (Mean values)

Time	MMS Ratio	MMS Significance ($p<0.01$)
0.3 hr vs 1 hr	1.14	-
0.3 hr vs 2 hrs	1.51	-
0.3 hr vs 4.5 hrs	9.98	+
0.3 hr vs 12 hrs	8.06	+

Time	MNUA Ratio	MNUA Significance ($p<0.01$)
0.3 hr vs 1 hr	1.14	-
0.3 hr vs 2 hrs	2.30	-
0.3 hr vs 4.5 hrs	1.02	-
0.3 hr vs 12 hrs	2.02	-

Part II: Comparison of means - 3MeA/7MeG ratio

Time	Calculated 't'	Significance ($p<0.01$)
MNUA - 4.5 hrs vs 12 hrs	3.11	+
MMS - 2 hrs vs 12 hrs	-7.24	+

Part III: Comparison of means - O^6MeG/7MeG ratio

Time	Calculated 't'	Significance ($p<0.01$)
4.5 hrs vs 12 hrs	3.15	+

Fig. 29: Mice treated with either (^{14}C)-methylnitrosourea (dose: 0.6 mM/Kg body weight) or (^{14}C)-methyl methanesulphonate (dose: 1.2 mM/Kg body weight) - DNA isolated from different organs at various intervals, hydrolysed and chromatographed. Shows the ratios of 3MeA/7MeG and of 0^6-MeG/7MeG (after normalization).

(Li = Liver; G = Gut; K = Kidney; Lu = Lung; T = Thymus; S = Spleen; BM = Bone marrow)

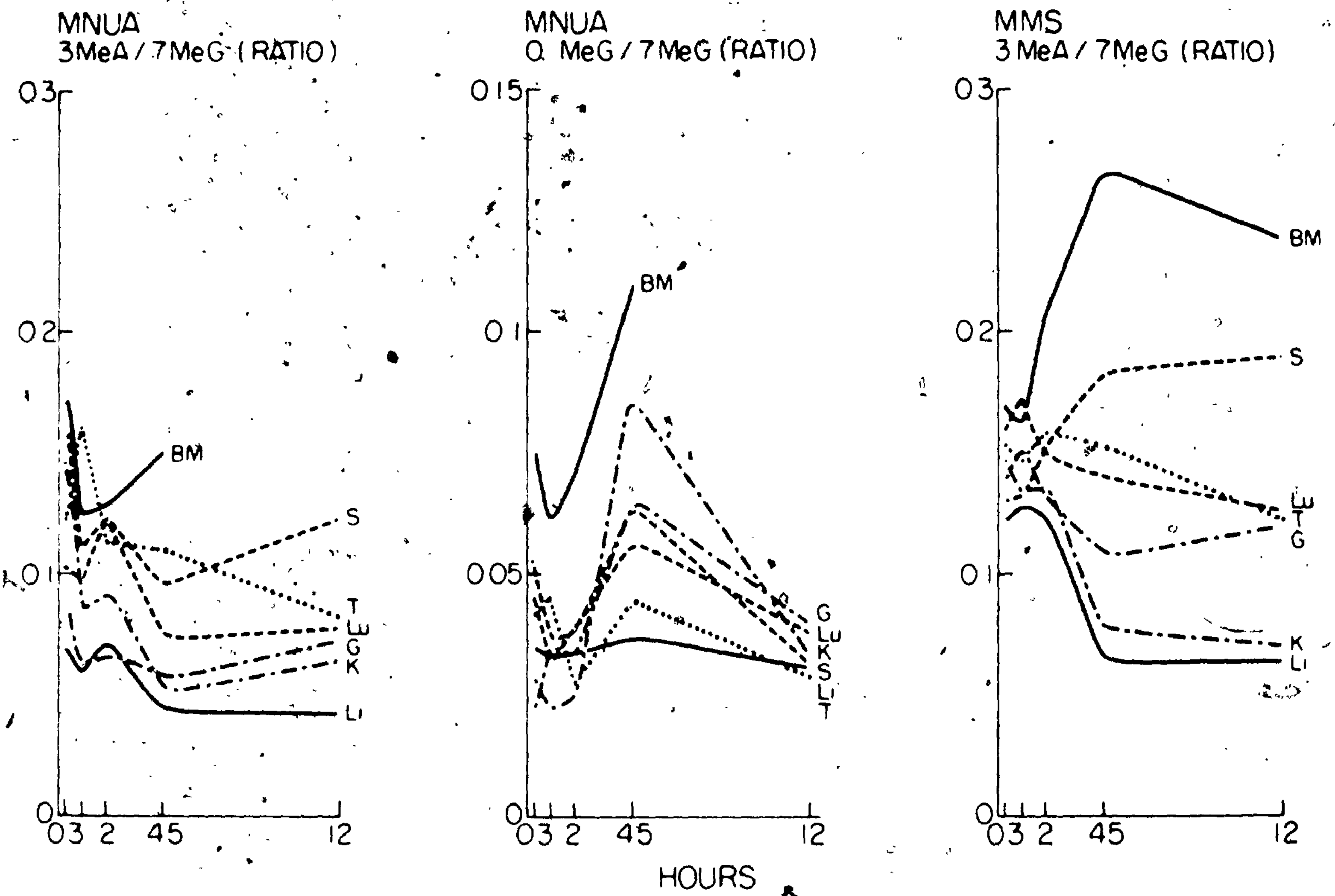


FIG.29

This approach demonstrates preferential removal of 3MeA over 7MeG and reveals significant tissue differences with respect to this capacity. It may be observed that the 3MeA/7MeG ratios for MMS are tightly clustered for all points and tissues during alkylation (first 2 hours), then the points diverge, showing marked fall in liver and kidney while in other tissues it declines slightly (in bone marrow and spleen the ratio rises) (Fig. 29). This is shown statistically in Table 37, where 3MeA/7MeG ratios for MMS show significant differences in variance at early vs late time confirming preferential removal of 3MeA and also suggesting a change in behaviour of tissues. This observation is indicative of a relative failure of a probable enzymic removal of 3MeA from DNA in tissues other than liver and kidney, particularly bone marrow and spleen. Such a failure could be due to a relative lack of the specific enzyme, or a partial inhibition of the enzyme, or inactivity of the enzyme during active cell cycle.

An examination of similar ratios for MNUA data does not lead to a clear-cut conclusion because of the tissue variations in overall DNA alkylation and thus of large standard deviations in the overall results (Table 37, Fig. 29). The MNUA results are consistent with preferential removal of 3MeA, but not statistically significantly so.

The comparison of the means of the 0^6-MeG/7MeG ratio after MNUA at peak and lower average points show a significant fall suggesting preferential removal of 0^6-MeG but the validity of this interpretation is uncertain because, as noted, the actual extent of 0^6-MeG formation and disappearance remains undetermined due to an uncertain amount of loss during hydrolysis.

Significance of the observations in carcinogenesis:

In terms of potential significance for the understanding of the mechanism of carcinogenesis in the murine system used, three observations emerge as the most important:

a) O^6-alkylation of guanine

b) probable lack of specific DNA repair in target tissues

c) the necessity for thymus and bone marrow regeneration in the genesis of thymic lymphomas.

The hypothesis that acquisition of a heritable change is a step in carcinogenesis is supported by the first two of these observations. MNUA is found to generate an easily measurable amount of O^6-MeG product in DNA of all tissues whereas MMS generates very little, if any, of it. MNUA is and MMS is not a carcinogen in the test system used. The finding of a difference in the generation of O^6-MeG is in agreement with previous observations that this base is formed in the nucleic acids by SN_1 agents which are also potent carcinogens and mutagens (Loveless, 1969; Lawley and Thatcher, 1970) and very little is formed by SN_2 agents with low oncogenic and mutagénic potencies (Lawley and Shah, 1972b).

Genetic studies of alkylation mutants in bacteria and bacteriophages have shown that most mutational events involved guanine to adenine transitions (Kreig, 1963; Osborn et al., 1967). According to the Watson-Crick model of DNA structure and replication, guanine would mispair with thymine only if there is no proton at N-1 site. Alkylation at N-7 position of guanine would increase the acidity of its N-1 atom, hence increase the proportion of proton deficient form (Lawley and Brookes, 1961), but this situation does not necessarily guarantee mispairing with

thymine. In fact, no evidence for miscoding by 7MeG have been found with RNA templates (Ludlum, 1970) or with DNA templates (Hendler et al., 1970). Further, the failure of MMS to induce transition mutation in extra-cellularly treated T-even phages cannot be satisfactorily explained although 7MeG is the bulk reaction product (Loveless and Hampton, 1969). Even in the mouse system, MMS has been shown to induce very few point mutations (Russell et al., 1970).

It appears, therefore, that the position of 7MeG as a mutagenic factor is not very firm; even if it is assumed that 7MeG is potentially mutagenic, the probability of its actually inducing mutation is low.

On the other hand, the formation of 0^6-MeG guarantees that the alkylated molecule would remain in the enol form with no proton at N-1 site, the ideal pre-condition for mispairing with thymine (Loveless, 1969). Thus, the probability of miscoding appears much greater with 0^6-MeG than with 7MeG.

Despite the fact that 0^6-MeG has the greater probability of inducing transition mutation, its role in carcinogenesis has not been satisfactorily established as yet, in that in this experimental work all tissues have this compound in their DNA following MNUA but only some become neoplastic, i.e., lung and thymus (with the assistance of bone marrow). It has also been shown that not all SN_1 agents are carcinogenic in this mouse system, e.g., MNNG (Frei and Joshi, 1973). It seems, therefore, that the formation of 0^6-MeG may be a necessary but not sufficient factor in neoplasia (cf., Craddock, 1973).

Kleihues and Magee (1973) have contrasted the effects of MMS and MNUA on rat nervous tissue tumour development, although both agents

induced tumours, only one, MNUA, induced O^6-MeG in brain DNA in their hands. It must be noted, however, that at comparable doses MNUA gave an over 90% incidence of brain tumours (Druckrey et al., 1965) while the incidence with MMS was 7% only (Swann and Magee, 1969). This is close to the twenty-fold difference in O^6-MeG production detected by Lawley and Shah (1972b) by MMS in contrast to SN_1 agents. It is, therefore, possible that exact quantitative relationships have to be established before the importance of O^6-alkylation of guanine is clarified. The present results support the contention that O^6-alkylation of guanine may be important.

Mammalian cells have been reported to repair damage to DNA induced by alkylating agents (Roberts et al., 1968; Hahn et al., 1968; Ayad et al., 1969; Lieberman et al., 1971; Roberts, 1972), but it is not fully established what damage is repaired, whether the original base sequence is restored or whether only the DNA template is restored just sufficient for replication to occur and for the cell to survive but with DNA still modified such as to lead to subsequent mutations. Since, however, as far as is known, repair follows MMS treatment as much as MNUA treatment, an observation with which the present results are consistent, an involvement of repair in carcinogenesis could more likely be in disturbances of repair. There could be a decrease or absence of repair, or a slowing of the repair process, both of which would favour the expression of the mutational potential of O^6-MeG, particularly in cycling tissues.

The contribution of the present work is the detection of a definite probability that the target tissues, at least for thymic lymphoma induction, lacked the ability for preferential removal of 3MeA

from their DNA. This indication of lack of DNA repair, in combination with O^6-MeG (and thus only following MNUA treatment) could increase the yield of forward mutations of the GC → AT type significantly. This observation therefore also supports the hypothesis that acquisition of a heritable change is a step in carcinogenesis.

Slowing of DNA repair may have some role in alkylation carcinogenesis as has been suggested by Damjanov et al. (1973). They studied the repair pattern of rat liver DNA after intraperitoneal injection of single carcinogenic doses of MMS (120 mg/Kg body weight), MNUA (80 mg/Kg body weight), DMN (10 mg/Kg body weight) and MAM (25 mg/Kg body weight) in partially hepatectomised animals and showed that repair is not complete even at 14 days following the two hepatocarcinogens, DMN and MAM, whereas it is complete by 48 hours after MMS (Cox et al., 1973) and by 6 days after MNUA (Damjanov et al., 1973). The present results have shown no clear-cut early decline in the levels of alkylation following MNUA (Table 38) in contrast to MMS. This is compatible with a possibility of slowing of DNA repair following MNUA, but not a good evidence for it.

As with bacterial systems (Witkin, 1969), post replication repair errors may also contribute to mutations by carcinogens, if there is a shift to this type of repair following carcinogen treatment (cf., Roberts, 1972). The present results shed no light on this alternative.

These possibilities suggest that more detailed studies of DNA repair in this system are needed.

That a combination of events need be present for tumours to develop, has been shown in the skin of mice (Berenblum and Shubik, 1949;

TABLE 38

Part I - Total alkylation of DNA following MNUA expressed as dpm/mg DNA/mCi/mmole of dose with mean, variance and standard deviation.

Tissue	At 0.3 hr	At 1 hr	At 2 hrs	At 4.5 hrs	At 12 hrs
Gut	2598	3072	3264	3704	2546
Liver	4421	3863	4133	6902	3913
Spleen	1644	1536	2038	1999	1468
Thymus	1451	1062	1487	2117	1659
Bone marrow	1518	1325	1647	1686	-
Lung	1614	2179	1747	2552	1430
Kidney	2280	2238	2874	3226	2128
$\overline{X}$	2218	2182	2456	3169	2191
V	1129006	1004528	985127	3213391	896030
s.d.	1063	1002	993	1793	947

Part II - Comparison of the means

Time	Calculated 't'	Significance ($p<0.01$)
0.3 hr vs 4.5 hrs	1.21	-
4.5 hrs vs 12 hrs	1.20	-

Boutwell, 1964). With skin carcinogens, one such event may be mutagenic and the other is always associated with some degree of hyperplasia though perhaps only sustained marked hyperplasia is effective (Frei and Stephens, 1968). With alkylating carcinogens too, strong evidences have been produced by Craddock (1971a; 1971b; 1973) that cell replication is a necessary factor in carcinogenesis in addition to the formation of 0^6-MeG. The present study strongly suggests this view. Only when the appropriate target tissues have undergone a significant degree of hyperplasia (while containing mutated cells perhaps) did tumours develop in tissues which initially had the 0^6-MeG product in their DNA. This was so specifically for the thymus and bone marrow during thymoma development, the third key observation in this work. The role of reparative hyperplasia in target tissues may be to multiply the miscoding error many times and thus enable some of the pre-neoplastic cells to form "critical sized clones" which according to Berenblum (1957) is essential for neoplastic manifestion.

From the viewpoint that a state of immunosuppression favours the survival and outgrowth of early tumour cells, there may be yet another difference between MNUA and MMS. Like many other alkylating agents capable of inducing tumours, a leukaemogenic dose of MNUA in neonatal mice was shown to cause marked and long lasting immunological deficit as assessed by splenic plaque forming cell determination and haemagglutinin titration (Parmiani et al., 1971).

Whether MNUA does reduce the immunological competence of these animals as judged by the above criteria was neither investigated in this system previously nor in the present study. It may be assumed that it

does, at least for a short period, particularly since it was shown to cause temporary thymolymphoid and myeloid degeneration.

MMS, on the other hand, failed to cause significant thymolymphoid and myeloid degeneration and no reports concerning its effects on the immunological functions are available at present.

No further study, also, was made in the present work of the role of the virus that is known to be somehow involved in the genesis of thymic lymphomas (Ball and McCarter, 1971; Frei et al., 1973).

V. SUMMARY

In order to isolate the causal events in the genesis of thymic lymphomas and pulmonary adenomas in mice, two monofunctional alkylating agents, methylnitrosourea and methyl methanesulphonate were injected intraperitoneally in inbred adult female Swiss mice and their effects studied and compared in seven different tissues at three levels:

1) their ability to induce tumours,
2) their ability to cause target tissue changes, and
3) their nature of reactions with DNA.

The stimulus for examining these two agents under the stated parameters stems from a number of previous observations with these two and a few other closely related agents:

a) The animals develop a low yield of apparently spontaneous pulmonary adenomas, breast carcinomas and thymic lymphomas.
b) Given in single doses intraperitoneally, methylnitrosourea and ethylnitrosourea induce a high yield of thymic lymphomas and pulmonary adenomas; ethyl methanesulphonate induces pulmonary adenomas only; methyl methanesulphonate and methylnitronitrosoguanidine do neither.
c) Fractionated doses of methylnitrosourea produce a higher yield of tumours than single doses.
d) Methylnitrosourea causes necrosis followed by regeneration in the thymolymphoid and myeloid tissues.
e) Methylnitrosourea causes immunosuppression.
f) Methylnitrosourea reacts with DNA in all tissues yielding mainly three products: 7-methylguanine, 3-methyladenine and O^6-methylguanine.

The present study reveals:

a) Methyl methanesulphonate, whether given in a single dose or in fractionated doses, is incapable of inducing tumours of any kind;

b) Methyl methanesulphonate, although it causes thymic weight loss fails to cause bone marrow tissue changes; similar results are obtained with two other agents, ethyl methanesulphonate and methylnitronitrosoguanidine, which are also incapable of inducing thymic lymphomas. In contrast, ethylnitrosourea, a thymic lymphoma inducer like methylnitrosourea, can cause both bone marrow and thymic changes;

c) In the study of the reaction products of DNA following use of radioactive agents it is essential to separate metabolically labelled normal bases from methylated bases especially in cycling tissues at advanced times;

d) In terms of the methylation products studied, the timing of peak level of methylation following methyl methanesulphonate agrees with available information about its biological half life; following methylnitrosourea, the timing of methylation levels remains undetermined;

e) On a molar basis, methyl methanesulphonate is less efficient in alkylation of all purine sites than methylnitrosourea;

f) Methylnitrosourea is followed by significant tissue differences in the level of 7-methylguanine and possibly also in the level of 0^6-methylguanine; the tissues known to develop tumours show lesser amounts of these two products;

g) Methylnitrosourea generates an easily measurable amount of O^6-methylguanine in all tissues, whereas methyl methanesulphonate generates very little of it, if it does at all;

h) Following methyl methanesulphonate, the changes in the 3-methyladenine/ 7-methylguanine ratio with time indicate preferential removal of 3-methyladenine from some tissues, in particular from liver and kidney and a lack of such removal from other tissues, particularly the bone marrow and spleen; on this point, methylnitrosourea data are not clear-cut but are consistent with the methyl methanesulphonate data;

i) On the basis of the present and past observations, the potential factors that may operate in alkylation carcinogenesis in inbred Swiss mice are:

Factors	Thymic Lymphomas	Pulmonary adenomas
1) Formation of O^6-alkylguanine (and the consequent induction of mutations)	+	+
2) Amount of O^6-alkylguanine formed	+	+
3) Abnormality of DNA repair in target tissues	+	±
4) Regeneration of target tissues	+	?
5) Immunosuppression	+	+
6) Role of virus	+	?

The murine system used in this work is well suited to the further study of these factors.

BIBLIOGRAPHY

Agarwal, M.K., and Weinstein, I.B. Modification of RNA by chemical carcinogens. II. In vivo reaction of N-acetylaminofluorene with rat liver RNA. Biochemistry, 9: 503-507, 1970.

Agosin, M., Cristen, R., Badinez, O., Gasic, G., Neghme, A., Pizzaro, O., and Jarpa, A. Cortisone-induced metastases of adenocarcinoma in mice. Proc. Soc. Exp. Biol. Med., 80: 128-131, 1952.

Austin, A.T. The action of nitrous acid on aliphatic amines. Nature, 188: 1086-1088, 1960.

Ayad, S.R., Fox, M., and Fox, B.W. Non-semiconservative incorporation of 5-bromo-2'-deoxyuridine in lymphoma cells treated with low doses of methyl methanesulphonate. Mutation Res., 8: 639-645, 1969.

Axel, R., Weinstein, I.B., and Farber, E. Patterns of transfer RNA in normal rat liver and during hepatic carcinogenesis. Proc. Natl. Acad. Sci. USA, 58: 1255-1260, 1967.

Ball, J.K., Huh, T.Y., and McCarter, J.A. On the statistical distribution of epidermal papillomata in mice. Brit. J. Cancer, 18: 120-123, 1964.

Ball, J.K., and McCarter, J.A. Role of bone marrow in induction of thymic lymphomas by neonatal injection of 7,12-dimethylbenz[a]anthracene. J. Natl. Cancer Inst., 41: 553-558, 1968.

Ball, J.K., and McCarter, J.A. Repeated demonstration of a mouse leukaemia virus after treatment with chemical carcinogens. J. Natl. Cancer Inst., 46: 751-762, 1971.

Ball, J.K., Sinclair, N.R., and McCarter, J.A. Prolonged immunosuppression and tumour induction by a chemical carcinogen injected at birth. Science, 152: 650-651, 1966.

3 OF/DE 3

ARC - 374

Baltimore, D. RNA-dependent DNA polymerase in virions of RNA tumour viruses. Nature, 226: 1209-1211, 1970.

Bannon, P., and Verly, W. Alkylation of phosphates and stability of phosphate triesters in DNA. Europ. J. Biochem., 31: 103-111, 1972.

Bauer, K.H. Mutationstheorie der Geschwulst-Eustechung. Ubergang von korperzellen in Geschwulstzellen durch Gen-Anderung. Springer - Verlag, Berlin, 1928.

Bauer, K.H. Das Krebsproblem. Springer - Verlag, Berlin, 1963.

Bautz-Freese, E. Transitions and trasversions induced by depurinating agents. Proc. Natl. Acad. Sci. USA, 47: 540-545, 1961.

Berenblum, I. Some recent advances in skin carcinogenesis. Ann. Roy. Coll. of Surgeons of Engl., 21: 339-357, 1957.

Berenblum, I., and Shubik, P. A new quantitative approach to the study of the stages of chemical carcinogenesis in the mouse's skin. Brit. J. Cancer, 3: 109-118, 1949.

Bergs, V.V., Bergs, M., and Chopra, H.C. A virus (RMTDV) derived from chemically induced rat mammary tumors. I. Isolation and general characteristics. J. Natl. Cancer Inst., 44: 913-921, 1970.

Block, M. Prelymphomatous or preleukaemic state in mice: Relation to human preleukaemia. Natl. Cancer Inst. Monogr., 22: 559-570, 1966.

Boutwell, R.K. Some biological aspects of skin carcinogenesis. Progr. Exptl. Tumor Res., 4: 207-250, 1964.

Boveri, T. The Origin of Malignant Tumours (first published Jena, 1914), Williams and Wilkins Co., Baltimore, USA, 1929.

Boyland, E., and Horning, E.S. The induction of tumours with nitrogen mustard. Brit. J. Cancer, 3: 118-123, 1949.

Britten, R.J., and Davidson, E.H. Gene regulation for higher cells. A theory. Science, 165: 349-357, 1969.

Brookes, P., and Lawley, P.D. The reaction of mono- and difunctional alkylating agents with nucleic acids. Biochem. J., 80: 496-503, 1961.

Brookes, P., and Lawley, P.D. Alkylating agents. Brit. Med. Bull., 20 (No 2): 91-95, 1964.

Brookes, P., and Lawley, P.D. In: Chemical Mutagens (A. Hollaender ed.) Vol. I., pp. 121-144, 1971, Plenum Press, N.Y.

Brown, D.D., and Blackley, A.W. Gene amplification proceeds by a chromosome copy mechanism. J. Mol. Biol., 63: 75-83, 1972.

Brown, D.M., and Todd, A.R. In: The Nucleic Acids (E. Chargaff and J.N. Davidson eds.) Vol. I, pp. 409-445, 1955, Academic Press, N.Y.

Burdon, R.H., and Smellie, R.M.S. RNA- and DNA-dependent RNA ploymerases of mammalian cells. Biochim. biophys. Acta, 61: 633-634, 1962.

Campbell, J.G. An investigation of the hepatotoxic effects in fowls of Ragwort (S. Jacobaea Linn) with special reference to the induction of liver tumors with Seneciphylline. Proc. Roy. Soc. Edinburgh, B., 66: 111, 1956.

Ceglowski, W.S., and Friedman, H. Immunosuppressive effects of Friend and Rauscher leukaemia disease viruses on cellular and humoral antibody formation. J. Natl. Cancer Inst., 40: 983-995, 1968.

Chakrabortty, H.C., and Burma, D.P. Multiple development in strip and circular chromatography. Analytica Chim. Acta, 15: 451-456, 1956.

Chan, G., Raincourt, M.W., Ceglowski, W.S., and Friedman, H. Leukaemia virus suppression of antibody-forming cells: Ultrastructure of infected spleens. Science, 159: 437-439, 1968.

Clapp, N.K., Craig, A.W., and Toya, R.E., Sr. Oncogenicity of Methyl methanesulphonate in male RF mice. Science, 161: 913-914, 1968.

Clayson, D.B. In: Chemical Carcinogenesis, pp. 412-417, 1962, J. & A. Churchill, Ltd., London.

Corbett, T.H., Heidelberger, C., and Dove, W.F. Determination of the mutagenic activity of bacteriophage T4 of Carcinogenic and non-carcinogenic compounds. Mol. Pharmacol., 6: 667-679, 1970.

Cox, R., Damjanov, I., Abanobi, S.E., and Sarma, D.S.R. A method for measuring DNA damage and repair in the liver in vivo. Cancer Res., 33: 2114-2121, 1973.

Craddock, V.M. Methylation of DNA in the intact animal and the effect of the carcinogens dimethylnitrosamine and ethionine. Biochim. biophys. Acta, 240: 376-389, 1971 (a).

Craddock, V.M. Liver carcinogens induced in rats by single administration of dimethylnitrosamine after partial hepatectomy. J. Natl. Cancer Inst., 47: 899-907, 1971 (b).

Craddock, V.M. The pattern of methylated purines formed in DNA of intact and regenerating liver of rats treated with the carcinogen dimethylnitrosamine. Biochim. biophys. Acta, 312: 202-210, 1973.

Craigie, D., and Bennett, J.H. Case of disease of the spleen, in which death took place in consequence of the presence of purulent material in the blood. Edinb. M. & S. J., 64: 400, 1845.
Quoted from Rich, M.A. Experimental leukaemia, pp. 1 - 13, 1968. Appleton-Centurey-Crofts, N.Y.

Cumming, R.B., and Walton, M.F. Fate and metabolism of some mutagenic alkylating agents in the mouse. I. Ethyl methanesulfonate and methyl methanesulfonate at sublethal dose in hybrid males. Mutation Res., 10: 365-377, 1970.

Damjanov, I., Cox, R., Sarma, D.S.R., and Farber, E. Patterns of damage and repair of liver DNA induced by carcinogenic methylating agents in vivo. Cancer Res., 33: 2122-2128, 1973.

Dickens, F., and Jones, H.E.H. Carcinogenic activity of a series of reactive lactones and related substances. Brit. J. Cancer, 15: 85-100, 1961.

Dipple A., Lawley, P.D., and Brookes, P. Theory of tumour initiation by chemical carcinogens: dependence of activity on structure of ultimate carcinogen. Europ. J. Cancer, 4: 493-506, 1968.

Druckrey, H., Preussmann, R., Schmahl, D., and Muller, M. Erzeugung von Magenkrebs durch Nitrosamide an Ratten. Naturwissenschaften, 48: 165, 1961.

Druckrey, H., Steinhoff, D., Preussmann, R., and Ivankovic, S. Krebserzeugung durch einmalige Dosis von Methylnitroso-harnstoff und verschiedenen Dialkyl-nitrosaminen. Naturwissenschaften, 50: 735, 1963.

Druckrey, H., Steinhoff, D., Preussmann, R., and Ivankovic, S. Erzeugung von Krebsdurch eine einmalige Dosis von Methylnitroso-

Harnstoff und verschiedenen Dialkylnitrosaminen an Ratten. Z. Krebsforsch, 66: 1-10, 1964 (a).

Druckrey, H., Preussmann, R., Ivankovic, S., Mennel, H.D., and Stahl, K.W. Selektive Erzeugung von Blasenkrebs an Ratten durch Dibutyl- und N-Butyl-N-butanol (4)-nitrosamin. Z. Krebsforsch, 66: 280-290, 1964(b).

Druckrey, H., Ivankovic, S., and Preussmann, R. Selektive Erzeugung maligner Tumoren un Gehrin und Ruckenmark von Ratten durch N-Methyl-N-nitrosoharnstoff. Z. Krebsforsch, 66: 389-408, 1965.

Druckrey, H., Preussmann, R., Ivankovic, S., and Schmahl, D. Organotrope carcinogene Wirkungen bei 65 verschiedenen N-Nitro-Verbinungen an BD-Ratten. Z. Krebsforsch, 69: 103-201, 1967.

Druckrey, H., Ivankovic, S., Bucheter, J., and Preussmann, R. Erzeugung von Magen- und Pankreas-Krebs beim Meerschweinchen durch Methylnitrosoharnstoff und -urethan. Z. Krebsforsch, 72: 167-182, 1968.

Druckrey, H., Preussmann, R., and Ivankovic, S. N-nitroso compounds in organotropic and transplacental carcinogenesis. Ann. N.Y. Acad. Sci., 2(Art 2): 676-696, 1969.

Dulbecco, R. Characteristics of virus-cell complexes. Amer. J. Med., 38: 669-677, 1965.

Dutton, A.H., and Heath, D.F. Demethylation of dimethylnitrosamine in rats and mice. Nature, 178: 644, 1956.

Eberth, C.J. Leukamis der Maus. Arch. Path. Anat., 72: 108-109, 1878. Quoted from Dunn, T.B. Normal and pathologic anatomy of the reticular tissue in laboratory mice with a classification and discussion of neoplasms. J. Natl. Cancer Inst., 14: 1281-1433, 1954.

Endo, H., Ishizawa, M., and Kamiya, T. Induction of bacteriophage formation in lysogenic bacteria by a potent carcinogen, 4-nitroquinoline 1-oxide, and its derivatives. Nature, 198: 195-196, 1963.

Farber, E. Biochemistry of carcinogenesis. Cancer Res., 28: 1859-1870, 1968.

Fink, K., and Adams, W.S. Paper chromatographic data for purines, pyrimidines and derivatives in a variety of solvents. J. Chromatog., 22: 118-129, 1966.

Fiore-Donati, L., Chieco-Bianchi, L., Benedictis, G., and Maiorano, G. Leukaemogenesis by urethan in Swiss new born mice. Nature, 190: 278-279, 1961.

Fox, M., and Ayad, S.R. Characteristics of repair synthesis in P388 cells treated with methyl methanesulphonate. Chem. -Biol. Interactions, 3: 193-211, 1971.

Frei, J.V. Toxicity, tissue changes, and tumour induction in inbred Swiss mice by methyl -nitrosamine and -amide compounds. Cancer Res., 30: 11-17, 1970.

Frei, J.V. Tumour induction by low molecular weight alkylating agents. Chem. -Biol. Interactions, 3: 117-121, 1971 (a).

Frei, J.V. Tissue-dependent differences in DNA methylation products of mice treated with methyl-labelled methylnitrosourea. Int. J. Cancer, 7: 436-442, 1971(b).

Frei, J.V., Iwasutiak, R., and Viragos, G. Lack of significant visible C-type virus activation in the bone marrow and thymus of mice given a leukaemogenic dose of methylnitrosourea. Chem. -Biol. Interactions, 6: 333-337, 1973.

Frei, J.V., and Joshi, V.V. Lack of induction of thymomas and of pulmonary adenomas in inbred Swiss mice by N-methyl-N'-nitro-N-nitrosoguanidine. Chem. -Biol. Interactions, (in press).

Frei, J.V., and Stephens, P. The correlation of promotion of tumor growth and of induction of hyperplasia in epidermal tumor stage carcinogenesis. Brit. J. Cancer, 12: 83-92, 1968.

Friend, C. Cell-free transmission in adult Swiss mice of a disease having the character of a leukaemia. J. Exp. Med., 105: 307-318, 1957.

Gardner, W.U., Dougherty, T.F., and Williams, W.L. Lymphoid tumours in mice receiving steroid hormones. Cancer Res., 4: 73-87, 1944.

Gatti, R.A., and Good, R.A. In: Oncology - Proceedings of the tenth international cancer congress, Vol. I, pp. 803-817, 1970. Year Book Medical Publishers, Inc., Chicago, U.S.A.

Gelboin, H.V. Carcinogens, Enzyme induction, and Gene action. Adv. Cancer Res., 10: 1-81, 1967.

Graffi, A., and Hoffmann, F. Starke leukaemogene Wirkung von N-methyl-N-nitrozo Harnstoff bei der Mans nach einmaliger Applikation an neugeborene Tiere. Acta Biol. Med. Germ., 17: K33-K35, 1966.

Graffi, A., Hoffmann, F., and Schütt, M. N-methyl-N-nitrosourea as a strong topical carcinogen when painted on the skin of rodents. Nature, 214: 611, 1967.

Green, H.N. An immunological concept of cancer: a preliminary report. Brit. Med. J., ii: 1374-1380, 1954.

Green, H.N. Immunologic basis of carcinogenesis. Brit. Med. Bull., 14: 101-105, 1958.

Griffin, A.C., Brandt, E.L., and Tatum, E.L. Induction of tumours with nitrogen mustards. Cancer Res., 11: 253, 1951.

Gross, L. Pathogenic properties, and "vertical" transmission of the mouse leukaemia agent. Proc. Soc. Exp. Biol. Med., 78: 342-348, 1951.

Gross, L. Development and serial cell-free passage of a highly potent strain of mouse leukaemia virus. Proc. Soc. Exp. Biol. Med., 94: 767-771, 1957.

Gryschek, G. Immunosuppressive effect of leukaemogenic substance of N-methyl-N-nitrosourea in the mouse. Acta. Biol. Med. Ger., 27: 195-200, 1971.

Haaland, M. Les tumeurs de la souris. Ann. Inst. Pasteur., 19: 165-208, 1905.

Haddow, A., Chemical carcinogens and their modes of action. Brit. Med. Bull., 14: 79-92, 1958.

Hahn, G., Yang, S.J., and Pareker, V. Repair of sublethal damage and unscheduled DNA synthesis in mammalian cells treated with monofunctional alkylating agents. Nature, 220: 1142-1144, 1968.

Haran-Ghera, N. A leukaemogenic filtrable agent from chemically-induced lymphoid leukemia in C57BL mice.
Proc. Soc. Exp. Biol. Med., 124: 697-699, 1967.

Harington, J. S. The sulphydryl group and carcinogenesis.
Adv. Cancer Res., 10: 247-309, 1967.

Heath, D.F., and Dutton, A. The detection of metabolic products from dimethylnitrosamine in rats and mice. Biochem. J., 70: 619-626, 1958.

Heath, D.F., and Jarvis, J.A.E. The polarographic determination of dimethylnitrosamine in animal tissues. The Analyst, 80: 613-616, 1955.

Hendler, S., Fürer, E., and Srinivasan, P.R. Synthesis and chemical properties of monomers and polymers containing 7-methylguanine and investigation of their substrate or template properties of bacterial DNA or RNA polymerases. Biochemistry, 9: 4141-4152, 1970.

Heppel, L.A. In: Methods in Enzymology (L. Grossman and K. Moldave eds.) Vol. XII, pp. 316-317, 1967, Academic Press, N.Y.

Herrold, K.M. Effect of route of administration of the carcinogenic action of diethylnitrosamine (N-nitrosodiethylamine). Brit. J. Cancer, 18: 763-767, 1964.

Heston, W.E. Induction of pulmonary tumours in strain A mice with methyl-bi[illegible]-chloroethyl)amine hydrochloride. J. Natl. Cancer Inst., 10: 125-130, 1949.

Heston, W.E. Occurrence of tumors in mice injected subcutaneously with sulphur mustard and nitrogen mustard. J. Natl. Cancer Inst., 14: 131-140, 1953.

Hiraki, S. Induction of malignant lymphomas by N,N'-dimethylnitrosourea in adult mice. Gann, 62: 135-137, 1971.

Hodgkin, T. On some morbid appearances of the absorbent glands and spleen. Med. Chir. Trans., 17: 68, 1832.
Quoted from Molander, D.W., and Pack, G.T. In: Hodgkin's disease, pp. 20-63, 1968, Charles C. Thomas, Springfield, Illinois, USA.

Hudson, R.F. Personal communication (as quoted by Ross, W.C.J. In: Biological alkylating agents, p. 6, 1962, Butterworth & Co. Ltd., London.

Huebner, R.J., and Todaro, G.J. Oncogenes of RNA tumour viruses as determinants of cancer. Proc. Natl. Acad. Sci. USA, 64: 1087-1094, 1969.

Hueper, W.C. Occupational Tumours and Allied Diseases. Charles C. Thomas, Springfield, Illinois, USA, 1942.

Hueper, W.C., Wiley, F.H., and Wolfe, H.D. Experimental production of bladder tumours in dogs by administration of Beta-naphthylamine. J. Ind. Hygiene Toxicol., 20: 46-84, 1938.

Igel, H.J., Huebner, R.J., Turner, H.C., Kotin, P., and Falk, H.L. Mouse leukaemia virus activation by chemical carcinogens. Science, 166: 1624-1626, 1969.

Ingold, C.K. Structure and Mechanism in Organic Chemistry, Cornell University Press, N.Y., 1953.

Structure and Mechanism in Organic Chemistry (2nd ed.), pp. 421-555, 1970, G. Bell and Sons, Edinburgh.

Irino, S., Ota, Z., Sezaki, T., and Zuzaki, K. Cell-free transmission of 20-methylcholanthrene-induced RF mouse leukaemia and electron microscopic demonstration of virus particles in its leukemic tissues. Gann, 54: 225-237, 1963.

Jacob, F., and Monod, J. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol., 3: 318-356, 1961.

Jänisch, W., and Schreiber, D. Experimentelle Hirngeschwülste bei Kaninchen nach Injektion von Methylnitrosoharnstoff. Naturwissenschaften, 54: 171-172, 1967.

Jeanes, A., Wise, C.S., and Dimler, R.J. Improved techniques in paper chromatography of carbohydrates. Anal. Chem., 23: 415-420, 1951.

Joshi, V.V. Pathogenesis of chemically induced malignant lymphoma in mice. Ph.D. thesis, 1970, University of Western Ontario, London, Canada.

Joshi, V.V., and Frei, J.V. Effects of dose and schedule of methylnitrosourea on incidence of malignant lymphoma in adult female mice. J. Natl. Cancer Inst., 45: 335-339, 1970.

Kaplan, H. On the natural history of murine leukaemias. Cancer Res., 27: 1325-1340, 1967.

Kay, E.R.M., Simmons, N.S., and Dounce, A.L. An improved preparation of sodium desoxyribonucleate. J. Amer. Chem. Soc., 74: 1724-1726, 1952.

Kelly, M.G., O'Gara, R.W., Adamson, R.H., Gadekar, K., Botkin, C.C., Reese, W.H., and Kerber, W.T. Induction of hepatic cell carcinomas in monkeys with N-nitrosodiethylamine. J. Natl. Cancer Inst., 36: 323-351, 1966.

Kelly, M.G., O'Gara, R.W., Yancey, S.T., and Botkin, C. Carcinogenicity of 1-Methyl-1-Nitrosourea in newborn mice and rats. J. Natl. Cancer Inst., 41: 619-626, 1968.

Kennaway, E.L. Further experiments on cancer-producing substances. Biochem. J., 24: 497-504, 1930.

Kirby, K.S. A new method for the isolation of ribonucleic acids from mammalian tissues. Biochem. J., 64: 405-408, 1956.

Kirby, K.S. A new method for the isolation of deoxyribonucleic acids: evidence on the nature of bonds between deoxyribonucleic acid and protein. Biochem. J., 66: 495-504, 1957.

Kirby, K.S. Preparation of some deoxyribonucleic acid-protein complexes from rat-liver homogenetes. Biochem. J., 70: 260-265, 1958.

Kirby, K.S. The preparation of deoxyribonucleic acids by the p-aminosalicylate-phenol method. Biochim. biophys. Acta, 36: 117-124, 1959.

Kirby, K.S. Separation of deoxyribonucleic acid from ribonucleic acid without the use of enzymes. Biochim. biophys. Acta, 47: 18-26, 1961.

Kirby, K.S. Deoxyribonucleic acids. IV. Preparation of deoxyribonucleic acid from Drosophila eggs. Biochim. biophys. Acta, 55: 382-384, 1962.

Kirby, K.S. Isolation of deoxyribonucleic acid and ribosomal ribonucleic acid from Escherichia coli. Biochem. J., 93: 5C-6C, 1964.

Kirby, K.S. Isolation and characterization of Ribosomal Ribonucleic Acid. Biochem. J., 96: 266-269, 1965.

Kirby, K.S. Deoxyribonucleic acids and cancer. In: Methods in Cancer Research, 3: 1-27, 1967.

Kirschbaum, A., Shapiro, J.R., and Mixer, H.W. Synergistic action of leukaemogenic agents. Cancer Res., 13: 262-268, 1953.

Kirschbaum, A., Strong, L.C., and Gardner, W.V. Influence of methylcholanthrene on age incidence of leukaemia in several strains of mice. Proc. Soc. Exp. Biol. Med., 45: 287-289, 1940.

Kit, S. Investigations of the DNA content, base composition and chromatography on ECTEOLA-cellulose of normal and tumour preparations. Arch. Biochem. Biophys., 87: 330-336, 1960.

Kleihues, P., and Magee, P.N. Alkylation of rat brain nucleic acids by N-methyl-N-nitrosourea and methyl methanesulphonate. J. Neurochem., 20: 595-606, 1973.

Krebs, C., Rask-Nielsen, H.C., and Wagner, A.C. The origin of lymphosarcomatosis and its relation to other forms of leukosis in white mice. Acta Radiol. (Suppl.), 10: 1-53, 1930.

Kreig, D.R. Ethyl methanesulphonate-induced reversion of bacteriophage T4 and rII mutants. Genetics, 48: 561-580, 1963.

Kundraat. Ueber lympho-sarkomatosis: Wien-lkin. Wchnschr., 6: 211-234, 1893. Quoted from Leavell, B.S., and Thorup, O.A. Fundamentals of haematology (2nd ed.), pp. 445-474, 1966. W. B. Saunders, Philadelphia, USA.

Kunii, A., Takemoto, N., and Furth, J. Leukaemogenic filtrable agent from oestrogen induced thymic lymphoma in RF mice. Proc. Soc. Exp. Biol. Med., 119: 1211-1215, 1965.

Kurland, C.G., and Maaloe, O. Regulation of Ribosomal and Transfer RNA Synthesis. J. Mol. Biol., 4: 193-210, 1962.

Law, L.W. The effect of gonadectomy and adrenalectomy on the appearance and incidence of spontaneous lymphoid leukaemia in mice. J. Natl. Cancer Inst., 8: 157-159, 1947.

Lawley, P.D. The action of alkylating mutagens and carcinogens on nucleic acids: N-methyl-N-nitroso compounds as methylating agents. In: Topics in chemical carcinogenesis (W. Nakahara, S. Takayama, T. Sujimura, and S. Odashima eds.), pp. 237-258, 1972, University of Tokyo Press, Japan & University Park Press, Baltimore, Md. USA.

Lawley, P.D. Reaction of N-methyl-N-nitrosourea (MNUA) with ^{32}P-labelled DNA: evidence for formation of phosphotriesters. Chem.-Biol. Interactions, 7: 127-130, 1973.

Lawley, P.D., and Brookes, P. Acidic dissociation of 7:9-dialkylguanines and its possible relation to mutagenic properties of alkylating

agents. Nature, 192: 1081-1082, 1961.

Lawley, P.D., and Brookes, P. Further studies on the alkylation of nucleic acids and their constituents nucleotides. Biochem. J., 89: 127-138, 1963.

Lawley, P.D., and Orr, D.J. Specific excision of methylation products from DNA of Escherichia coli treated with N-methyl-N'-nitro-N-nitrosoguanidine. Chem. -Biol. Interactions, 2: 154-157, 1970.

Lawley, P.D., Orr, D.J., and Shah, S.A. Reaction of alkylating mutagens and carcinogens with nucleic acids: N-3 of guanine as a site of alkylation by N-methyl-N-nitrosourea and dimethyl sulphate. Chem. -Biol. Interactions, 4: 431-434, 1971.

Lawley, P.D., and Shah, S.A. Methylation of RNA by dimethyl sulphate, methyl methanesulphonate, N-methyl-N-nitrosourea, N-methyl-N'-nitro-N-nitrosoguanidine - comparison of chemical analyses at the nucleoside and base levels. Biochem. J., 128: 117-132, 1972 (a).

Lawley, P.D., and Shah, S.A. Reaction of alkylating mutagens and carcinogens with nucleic acids: detection and estimation of a small extent of methylation at O-6 of guanine in DNA by methyl methanesulphonate in vitro. Chem. -Biol. Interactions, 5: 286-288, 1972 (b).

Lawley, P.D., and Shah, S.A. Methylation of DNA by ^{3}H-^{14}C-methyl-labelled N-methyl-N-nitrosourea - evidence for transfer of the intact methyl group. Chem. -Biol. Interactions, 7: 115-120, 1973.

Lawley, P.D., and Thatcher, C.J. Methylation of DNA in cultured mammalian cells by N-methyl-N'-nitro-N-nitrosoguanidine: the influence of cellular thiol concentrations on the extent of methylation and the N-Oxygen atom of guanine as a site of methylation. Biochem. J., 116: 693-697, 1970.

Leaver, D.D., Swann, P.F., and Magee, P.N. The induction of tumours in the rat by a single oral dose of N-nitrosomethylurea. Brit. J. Cancer, 23: 177-187, 1969.

Lett, J.T., Parkins, G.M., and Alexander, P. Physicochemical changes produced by ionizing radiations and by the alkylating agents: evidence for a similar mechanism at the molecular level. Arch. Biochem., 97: 80-93, 1962.

Lieberman, M.W., Baney, R.N., Lee, R.E., Sell, S., and Farber, E. Studies on the DNA repair in human lymphocytes treated with proximate carcinogens and alkylating agents. Cancer Res., 31: 1307-1312, 1971.

Lieberman, M., and Kaplan, H.S. Leukaemogenic activity of filtrates from radiation induced lymphoid tumours of mice. Science, 130: 387-388, 1959.

Lijinsky, W., Loo, J., and Ross, A.E. Mechanism of alkylation of nucleic acids by nitrosodimethylamine. Nautre, 218: 1174-1175, 1968.

Lijinsky, W., Garcia, H., Keefer, L., Loo, J., and Ross, A.E. Carcinogenesis and alkylation of rat liver nucleic acids by nitrosomethylurea and nitrosoethylurea administered by intraportal injection. Cancer Res., 32: 893-897, 1972.

Lingens, F., Haerlin, F., and Sussmuth, R. Mechanism of mutagenesis by MNNG: methylation of nucleic acids by CD_3-labelled MNNG in presence of cysteine and in cells of Escherichia coli. FEBS Lett., 13: 241-242, 1971.

Loring, H.S. In: The Nucleic Acids (E. Chargaff and J.N. Davidson eds.), Vol. I, pp. 191-209, 1955, Academic Press, N.Y.

Loveless, A. Genetic and Allied Effects of Alkylating Agents, 1966, Butterworth & Co. Ltd., London.

Loveless, A. Possible relevance of 0-6 alkylation of deoxyguanosine to the mutagenicity and carcinogenicity of nitrosamines and nitrosamides. Nature, 223: 206-207, 1969.

Loveless, A., and Hampton, C.L. Inactivation and mutation of coliphage T2 by N-methyl- and N-ethyl-N-nitrosourea. Mutation Res., 7: 1-12, 1969.

Ludlum, D.B. Alkylated polycytidylic acid templates for RNA polymerase. Biochim. biophys. Acta, 213: 142-148, 1970.

Ludlum, D.B. Methylated polydeoxyribocytidylic acid templates for RNA polymerase. Biochim. biophys. Acta, 247: 412-418, 1971.

Ludlum, D.B., and Wilhelm, R.C. Ribonucleic acid polymerase reactions with methylated polycytidylic acid templates. J. Biol. Chem., 243: 2750-2753, 1968.

Lwoff, A. Lysogeny. Bact. Rev., 17: 269-337, 1953,

Magee, P.N. Toxic liver injury. The metabolism of dimethylnitrosamine. Biochem. J., 64: 676-682, 1956.

Magee, P.N. The possible significance of alkylation in carcinogenesis. N. Z. med. J., 67: 59-72, 1968.

Magee, P.N. In vivo reactions of nitroso compounds. Ann. N.Y. Acad. Sci., 163 (Art 2): 717-730, 1969.

Magee, P.N., and Barnes, J.M. The production of malignant primary hepatic tumours in the rat by feeding dimethylnitrosamine. Brit. J. Cancer 10: 114-122, 1956.

Magee, P.N., and Barnes, J.M. Induction of kidney tumours in the rat with dimethylnitrosamine (N-nitrosodimethylamine). J. Path. Bact., 84: 19-31, 1962.

Magee, P.N., and Barnes, J.M. Carcinogenic Nitroso Compounds. Advan. Cancer Res., 10: 163-246, 1967.

Magee, P.N., and Farber, E. Toxic liver injury and carcinogenesis. Methylation of rat-liver nucleic acids by dimethylnitrosamine in vivo. Biochem. J., 83: 114-124, 1962.

Magee, P.N., Swann, P.F. Nitroso compounds. Brit. Med. Bull., 25: 240-244, 1969.

Magee, P.N., and Vandekar, M. Toxic liver injury The metabolism of dimethylnitrosamine in vitro. Biochem. J., 70: 600-605, 1958.

Malmgren, R.A., Bennison, B.E., and McKinley, T.W. Jr. Reduced antibody titers in mice treated with carcinogenic and other cancer chemotherapeutic agents. Proc. Soc. Exp. Biol. Med., 79: 484-488, 1952.

Margison, G.P., Capps, M.J., O'Connor, P.J., and Craig, A.W. Loss of 7-methylguanine from rat liver DNA after methylation in vivo with methyl methanesulphonate or dimethylnitrosamine. Chem.-Biol. Interactions, 6: 119-124, 1973.

Marie, P., Clunet, J., and Raulot-Lapointe, G. Nouveau cas de tumour maligne provoquée par une radiodermite experimentale dans le rat blanc. Bull. Ass. Franc. Etude Cancer, 5: 125-135, 1912.

Marko, A.M., and Butler, G.C. The isolation of sodium desoxyribonucleate with sodium dodecyl sulfate. J. Biol. Chem., 190: 165-176, 1951.

Marshak, A., and Vogel, H.J. Quantitative micro-analysis of nucleic acid purines and pyrimidines without prior isolation of the nucleic acids. Federation Proc., 9: 85-86, 1950.

McCalla, D.R., Reuvers, A., and Kitai, R. Inactivation of biologically active N-methyl-N-nitroso compounds in aqueous solution: effect of various conditions of pH and illumination. Can. J. Biochem., 46: 807-811, 1968.

Medvedev, Z.A. Possible role of repeated nucleotide sequences in DNA in the evolution of life spans of differentiated cells. Nature, 237: 453-454, 1972.

Metcalf, D. Histologic and transplantation studies on preleukaemic thymus of the AKR mouse. J. Natl. Cancer Inst., 37: 425-442, 1966(a).

Metcalf, D. The thymus. New York: Springer-Verlag, 89-117, 1966 (b).

Miller, E.C., and Miller, J.A. Mechanisms of chemical carcinogens: Nature of proximate carcinogens and interactions with macromolecules. Pharmacol. Rev., 18: 805-838, 1966.

Moloney, J.B. Preliminary studies on a mouse lymphoid leukaemia virus extracted from sarcoma 37. Proc. Amer. Assoc. Cancer Res., 3: 44, 1959.

Monod, J., Changeux, J.P., and Jacob, F. Allosteric proteins and cellular control systems. J. Mol. Biol., 6: 306-329, 1963.

Montagnier, L., and Sanders, F.K. Replicative form of encephalomyocarditis virus ribonucleic acid. Nature, 199: 664-667, 1963.

Murray, A., and Williams, D.L. Diazomethane-C. In: Organic syntheses with isotopes, Vol. I, pp. 583-585, 1958.

Nagata, C.A., Imamura, H.S., and Fukui, K. Changes of II-electron distribution of deoxyribonucleic acid after alkylation and their possible relation to the biological effect. Gann, 54: 109-117, 1963.

Neumann, E. Ueber myelozene Leukamic Berl. Klin. Wchnschr. 15: 69-72, 87-91, 115-119, 131-135, 1870.

Quoted from Rich, M.A. Experimental leukaemia, pp. 1-14, 1968, Appleton-Century-Crofts, New York.

Nishizuka, Y., and Shisa, H. Enhancement of 7,12-dimethylbenz[a] anthracene leukaemogenesis in mice by neonatal injection of cortisone acetate. Brit. J. Cancer, 12: 290-295, 1968.

Oberling, C. The riddle of cancer, p. 10, 1944, Yale University Press, New Haven, USA.

O'Connor, P.J., Capps, M.J., and Craig, A.W. Comparative studies of the hepatocarcinogen N,N-dimethylnitrosamine in vivo: reaction sites in rat liver DNA and the significance of their relative stabilities. Brit. J. Cancer, 27: 153-166, 1973.

O'Connor, P.J., Capps, M.J., Craig, A.W., Lawley, P.D., and Shah, S.A. Differences in the patterns of methylation in rat liver ribosomal ribonucleic acid after reaction in vivo with methyl methanesulphonate and NN-dimethylnitrosamine. Biochem. J., 129: 519-528, 1972.

Olson, A.O., and McCalla, D.R. Excision of 7-methylguanine from the DNA of Euglena gracilis. Biochim. biophys. Acta, 186: 229-231, 1969.

Ortwerth, B.Y., and Novelli, G.D. Studies on the incorporation of L-ethionine-ethyl-1-^{14}C into the transfer RNA of rat liver. Cancer Res., 29: 380-390, 1969.

Osborn, M., Person, S., Phillips, S., and Funk, F. A determination of mutagen specificity in bacteria using nonsense mutants of bacteriophage T4. J. Mol. Biol., 26: 437-447, 1967.

Painter, R., and Cleaver, J. Repair replication in HeLa cells after large doses of X-irradiation. Nature, 217: 369-370, 1967.

Paris, J.A. Pharmacologia, London, 1820.

Quoted from Daudel, P., and Daudel, R. In: Chemical Carcinogenesis and Molecular Biology, p. 4, 1966, Interscience Publishers, N.Y., USA.

Parmiani, G., Colnaghi, M.I., and Porta, G.D. Immuno-depression during urethane and N-nitrosomethylurea leukaemogenesis in mice. Brit. J. Cancer, 25: 354-364, 1971.

Pettijohn, D., and Hanawalt, P.C. Evidence for repair-replication of ultraviolet damaged DNA in bacteria. J. Mol. Biol., 9: 395-409, 1964.

Pillinger, D.J., Fox, B.W., and Craig, A.W. Metabolic studies in rodents with C^{14}-labeled Methyl methanesulfonate. In: Isotopes in Experimental Paharmacology, pp. 415-432, 1965, The University of Chicago Press, Chicago, USA.

Pitot, H.C., and Heidelberger, C. Metabolic regulatory circuits and carcinogenesis. Cancer Res., 23: 1694-1700, 1963.

Pott, P. London, 1775.

A reproduction of a communication of this author appears in Conference on biology of cutaneous cancer. J. Natl. Cancer Inst. Monogr., pp. 13, 1963.

Potter, J.S., and Richter, M.N. Studies on mouse leukaemia. VI. The predominating cell type in line I. Proc. Natl. Acad. Sci. USA, 18: 298-303, 1932.

Potter, J.S., Victor, J., and Ward, M.A. Histological changes preceding spontaneous lymphatic leukaemic in mice. Amer. J. Path. 19: 239-253, 1943.

Prehn, R.T. The role of immune mechanisms in the biology of chemically and physically induced tumours. In: Conceptual advances in immunology and oncology, pp. 475-486, 1963, Harper and Row, N.Y.

Preussmann, R., Druckrey, H., Ivankovic, S., and Hodenberg, A.V. Chemical structure and carcinogenicity of aliphatic hydrazo, azo and azoxy compounds and of triazenes, potential in vivo alkylating agents. Ann. N.Y. Acad. Sci., 163 (Art 2): 697-716; 1969.

Price, C.C., Gaucher, G.M., Koneru, P., Shibakawa, R., Sowa, J.R., and Yamaguchi, M. 'Relative reactivities for monofunctional nitrogen mustard alkylation of nucleic acid components. Biochim. biophys. Acta, 166: 327-359, 1968.

Quenouille, M.H. Introductory Statistics, p. 90, 1969, Pergamon Press, London.

Rappaport, H., and Baroni, C. A study of pathogenesis of malignant lymphoma induced in Swiss mouse by 7,12-dimethylbenz[a]anthracene injected at birth. Cancer Res., 22: 1067-1074, 1962.

Rauscher, F.J. A virus-induced disease of mice characterized by erythrocytopoiesis and lymphoid leukaemia. J. Natl. Cancer Inst., 29: 515-543, 1962.

Ribachi, R., and Giraldo, G. Leukemia virus release in chemically or physically induced lymphomas in BALB/c mice. Natl. Cancer Inst. Monogr., 22: 701-711, 1966.

Rich, [illegible].A. Experimental leukaemia, pp. 15-50, 1968, Appleton-Century-Crofts, New York.

Roberts, J.J. Repair of alkylated DNA in mammalian cells. Johns Hopkins Med. J., 1: Suppl. 229-238, 1972.

Roberts, J., Crathorn, A., and Brent. T. Repair of alkyated DNA in mammalian cells. Nature, 218: 970-972, 1968.

Roberts, J.J., Pascoe, J.M., Smith, B.A., and Crathorn, A.R. Quantitative aspects of the repair of alkylated DNA in cultured mammalian cells. II. Non-semiconservative DNA synthesis (repair synthesis) in HeLa and chinese hamster cells following treatment with alkylating agents. Chem. -Biol. Interactions, 3: 49-68, 1971.

Ross, W.C.J. Biological Alkylating Agents, pp. 64-92, 1962, Butterworth & Co. Ltd., London.

Rous, P. A transmissible avian neoplasm (Sarcoma of the common fowl). J. Exp. Med., 12: 696-705, 1910.

Rüdiger, R., and Rüdiger, H. Zur Berechnung optimaler Trennungsbedingungen für zwei stoffe mit bekkanten R_F-Werten bei mehrfach wiederholter Papierchromatographic. J. Chromatog., 17: 186-187, 1965.

Russell, W.L., Huff, S.W., and Gottlieb, D.J. The insignificant rate of induction of specific - locus mutations by five alkylating agents that produce high incidences of dominant lethality. Biol. Div. Oak Ridge National Laboratory (Tennessee), Ann. Rept., 1970.

Salaman, M.H., and Wedderburn, N. The immunosuppressive effect of Friend virus. Immunology, 10: 445-458, 1966.

Sasaki, T., and Yoshida, T. Experimentelle Erzeugung des Leberkarzinoms durch Futterung mit o-Amidoazotoluol. Virch. Arch., 295: 175, 1935. Quoted from Oberling, C. In: The riddle of cancer, p. 182, 1944. Yale University Press, New Haven, USA.

Schmähl, D., and Osswald, H. Carcinogenesis in different animal species by diethylnitrosamine. Experientia, 23: 497-498, 1967.

Schoental, R., and Bensted, J.P.M. Effects of whole body irradiation and partial hepatectomy on the liver lesions induced in rats by single dose of Retrorsine, a pyrrolizidine (Senecio) alkaloid. Brit. J. Cancer, 17: 242-251, 1963.

Schreiber, D., and Jänisch, W. Geschwulste bei Ratten nach wiederholter Applikation von N-Methyl-N-nitrosoharnstoff durch die Magensonde. Exp. Path., Bd.1: s 331-338, 1967.

Serebryanyi, A.M., and Mnatsakanean, R.M. A study of the molecular mechanism of the mutagenic action of N-nitoso-N-alkylurea. Carbamoylation of nucleosides by N-nitroso-N-alkylureas. FEBS Lett., 28: 191-194, 1972.

Siegler, R., and Rich, M.A. Pathogenesis of reticulum cell sarcoma in mice. J. Natl. Cancer Inst., 41: 125-143, 1968.

Smith, J.D., and Markham, R. Chromatographic studies on nucleic acids. 2. The quantitative analysis of ribonucleic acids. Biochem. J., 46: 509-513, 1950.

Sneen, R.A., and Larsen, J.W. Substitution at a saturated carbon atom. X. Unification of mechanisms SN_1 and SN_2. J. Amer. Chem. Soc., 91: 362-366, 1969.

Stein, W.H., and Moore, S. Chemical reactions of mustard gas and related compounds. III. The reaction of mustard gas with methionine. J. Org. Chem., 11: 681-685, 1946.

Stjernsward, J. Immunosuppressive effect of 3-methylcholanthrene. Antibody formation at the cellular level and reaction against weak antigenic homografts. J. Natl. Cancer Inst., 35: 885-892, 1965.

Stjernsward, J. Effect of noncarcinogenic and carcinogenic hydrocarbons on antibody-forming cells measured at the cellular level in vitro. J. Natl. Cancer Inst., 36: 1189-1195, 1966.

Stjernsward, J. Further immunological studies of chemical carcinogenesis. J. Natl. Cancer Inst., 38: 515-526, 1967.

Strauss, B., Coyle, M., and Robbins, M. Consequences of alkylation for the behaviour of DNA. Ann. N.Y. Acad. Sci., 163 (Art 2): 765-787, 1969.

Stroobandt, G., and Brucher, J.M. Eteude de tumeurs nerveuses obtenues par l'administration de méthylnitrosourée an rat. Neurochirurgie, 14: 515-535, 1968.

Sulzberger, M.B., Herrman, F., Piccagli, R., and Frank, L. Incidence of epidermal methylcholanthrene tumours in mice after administraion of cortisone. Proc. Soc. Exp. Biol. Med., 82: 673-675, 1963.

Swann, P.F. The rate of breakdown of methyl methanesulphonate, dimethyl sulphate and N-methyl-N-nitrosourea in the rat. Biochem. J., 110: 49-52, 1968.

Swann, P.F., and Magee, P.N. Induction of rat kidney tumours by Ethyl methanesulphonate and nervous tissue tumours by Methyl methanesulphonate. Nature, 223: 947-949, 1969.

Swenberg, J.A., Koestner, A., and Wolfgang, W. The induction of tumours of the nervous system with intravenous methylnitrosourea. Lab. Invest., 26: 74-85, 1972.

Tamm, C., Hodes, M.E., and Chargaff, E. The formation of apurinic acid from the desoxyribonucleic acid of calf thymus. J. Biol. Chem., 195: 49-63, 1952.

Teitz, Y., Lennette, E.H., Oshiro, L.S., and Cremer, N.E. Release of C-type particles from normal rat thymus cultures and those injected with Moloney leukemia virus. J. Natl. Cancer Inst., 46: 11-23, 1971.

Temin, H.M. The protovirus hypothesis: speculation of the significance of RNA-directed DNA synthesis for normal development and for carcinogenesis. J. Natl. Cancer Inst., 46: III-VII, 1971.

Temin, H.M., and Mizutani, S. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature, 226: 1211-1213, 1970.

Terracini, B., and Stramignoni, A. Malignant lymphomas and renal changes in Swiss mice given methylnitrosourea. Europ. J. Cancer, 3: 435-436, 1967.

Thatcher, C.M. Chemical, Toxic, and Transforming Activities of N-Alkyl-N-Nitroso Compounds. Ph.D. thesis, 1971, pp. 25, 27, 36, The University of Western Ontario, London, Canada.

Thoma, J.A. Application and theory of unidimensional multiple chromatography. Anal. Chem., 35: 214-224, 1963(a).

Thoma, J.A. A quantitative approach to unidimensional multiple chromatography. J. Chromatog., 12: 441-452, 1963(b).

Thomas, C., Sierra, J.L., and Kersting, G. Hirntumoren bei Ratten nach oraler Gabe von N-Nitroso-N-Methyl-harnstoff. Naturwissenschaften, 54: 228, 1967.

Torre, G.D., and Porta, G.D. Type C viruses in the pancreas of normal C57BL mice. Cancer Res., 32: 1595-1597, 1972.

Toth, B. Development of malignant lymphomas by cell-free filtrates prepared from a chemically induced mouse lymphoma. Proc. Soc. Exp. Biol. Med., 112: 873-875, 1963.

Toth, B., Rappaport, H., and Shubik, P. Accelerated development of malignant lymphomas in AKR mice injected at birth with 7,12-dimethylbenz[a]anthracene. Proc. Soc. Exp. Biol. Med., 10: 881-884, 1962.

Trams, E.G., Nadkarni, M.V., Dequattro, V., Maengwyn-Davis, G.D., and Smith, P.K. Dimethanesulphoxybutane (myleran), preliminary studies on distribution and metabolic fate in the rat. Biochem. Pharmacol., 2: 7-16, 1959.

Tsutsui, H. Über das Künstlich erzeugte Cancroid bei der Maus. Gann, 12: 17-21, 1918.

Van Duuren, B.L., Nelson, N., Orris, L., Palmes, E.D., and Schmitt, F.L. Carcinogenicity of epoxides, lactones, and peroxy compounds. J. Natl. Cancer Inst., 31: 41-55, 1963.

Van Duuren, B.L., Orris, L., and Nelson, N. Carcinogenicity of epoxides, lactones, and peroxy compounds. II. J. Natl. Cancer Inst., 35: 707-717, 1965.

Van Durren, B.L., Langseth, L., Orris, L., Teebor, G., Nelson, N., and Kuschner, M. Carcinogenicity of epoxides, lactones, and peroxy compounds. IV. Tumour response in epithelial and connective tissue in mice and rats. J. Natl. Cancer Inst., 37: 825-838, 1966.

Van Duuren, B.L., Langseth, L., Orris, L., Baden, M., and Kuschner, M. Carcinogenicity of epoxides, lactones, and peroxy compounds. V. Subcutaneous injection in rats. J. Natl. Cancer Inst., 39: 1213-1216, 1967(a).

Van Duuren, B.L., Langseth, L., Goldschmidt, B.M., and Orris, L. Carcinogenicity of epoxides, lactones, and peroxy compounds. VI. Structure and carcinogenic activity. J. Natl. Cancer Inst., 39: 1217-1228, 1967(b).

Virchow, R. Weisses Blut und Milztumoren. Med. Ztg., 15: 157-163, 1846. Quoted from Leavell, B.S., and Thorup, O.A. Fundamentals of clinical haematology, 1966, W.B. Saunders, Philadelphia, USA.

Vischer, E., and Chargaff, E. The separation and quantitative estimation of purines and pyrimidines in minute amounts. J. Biol.Chem., 176: 703-714, 1948.

Walburg, H.E., Jr. Methylcholanthrene-induced neoplasms in germ free RFM mice. Int. J. Cancer, 8: 338-343, 1971.

Walpole, A.L., Roberts, D.C., Rose, F.L., Hendry, J.A., and Homer, R.F. Cytotoxic agents: IV. The carcinogenic actions of some monofunctional ethyleneimine derivatives. Brit. J. Pharmacol., 9: 306-323, 1954.

Watkins, W.M., and Wormall, A. The immunological properties of proteins treated with Di-2-chloroethylmethylamine. Biochem. J., 51: 577-583, 1952.

Weinstein, I.B. Modifications in transfer RNA during chemical carcinogenesis. In: Genetic Concepts and Neoplasia, pp. 380-408, 1969, Williams and Wilkins, Baltimore, USA.

Windmueller, H.G., Ackerman, C.J., and Engel, R.W. Reaction of ethylene oxide with histidine, methionine, and cysteine. J. Biol. Chem., 234: 895-899, 1959.

Witkin, E.M. Ultraviolet-induced mutations and DNA repair. Ann. Rev. Microbiol., 23: 487-514, 1969.

Wyatt, G.R. In: The Nucleic Acids (E. Chargaff and J.N. Davidson eds.) Vol. I, pp. 243-265, 1955, Academic Press, New York.

Wyatt, G.R., and Cohen, S.S. The bases of the nucleic acids of some bacterial and animal viruses: the occurrence of 5-Hydroxymethylcytosine. Biochem. J., 55: 774-782, 1953.

Yamagiwa, K., and Ichikawa, K. Über die künstliche Erzeugung von Carcinom (IV-te Mitteilung). Gann, 11: 19-26, 1917.

Yasuhira, K. Induction of malignant lymphoma and other tumours during experiments with anthralin painting of mice. Gann, 59: 195-200, 1968.

Zimmermans, A., Bentvezen, P., Hageman, P.C., and Calafat, J. Activation of a mammary tumor virus in 020 strain mice by X-irradiation and urethane. J. Gen. Virol., 4: 619-621, 1969.