NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

The Effect of Tin on the Structure and Function of Deoxyribonucleic Acid in Mammalian Cells.

by

John R.N. McLean

A Thesis submitted to the School of Graduate Studies of the University of Ottawa, as partial fulfillment of the requirements for the degree of Ph.D. in Biology.

University of Ottawa
Ottawa, Canada, 1981.

ABSTRACT

J.R. McLean

Radiation Protection Bureau, Health & Welfare
Ottawa, Ontario

Tin(Sn) is a heavy metal that accumulates in the environment as a result of industrial activity. Its concentration has increased 10-20 times during the period 1900-1972, with most of the increase occurring in the decade 1962-1972, before the era of stannous fluoride in toothpastes and stannous chloride in radiopharmaceuticals. Little, however, is known about the sub-clinical toxicity of tin, especially stannous (II) tin, which is the subject of this investigation.

Tin accumulates rapidly in tissues following exposure and is retained in the body with a long biological half-life. Microbeam analysis of blood cells treated with Sn(II) and Sn(IV) indicates that only the Sn(II) penetrates all of the cell types studied and is uniformly distributed throughout the cytoplasm and the nucleus. In some cells colloidal sized particles of Sn(II) can be identified in the cytoplasm.

Injection of stannous (II) chloride into mice at doses ≤ 7 mg Kg^{-1} inhibits deoxyribonucleic acid (DNA) synthesis in cells of the spleen and bone marrow and suppresses the primary immune responses. DNA damage is detected in the spleen cells of mice treated in vivo with an intraperitoneal dose of 7 mg Kg^{-1} Sn(II) up to 24 hours after administration. In vitro studies using Fluorescence analysis of DNA unwinding (FADU) and alkaline sucrose gradients (ASG), confirm that Sn (II) is damaging to the DNA of human peripheral blood lymphocytes (HPBL) and chinese hamster ovary (CHO) cells. The treatment of HPBL with Sn(II) at 10μM induces the same number of DNA lesions, measured as total strand breaks, as an exposure to 0.3 Gy of Colbalt-60 gamma rays. Complexes of Sn(II) methylene diphosphonate (MDP) and Sn(II) trans - 1,2-diaminocyclohexane N,N,N^1, N^1 tetraacetic acid (CDTA) are more efficient than Sn(II) chloride in producing chromosome breaks. The presence of 50μM Sn(II) in the incubation medium inhibits strand break repair by 75% in HPBL.

Stannous (II) chloride causes a rapid and prolonged suppression of DNA synthesis in lymphocytes that are stimulated by conconavalin A and in a transformed lymphoid cell line. A brief treatment of mouse or human lymphocytes with 25μM Sn(II) is sufficient to inhibit the transformed cell line by 50%. It is postulated that Sn(II) exerts its effect on cells by causing DNA damage which inhibits DNA synthesis in vitro and in vivo.

Résumé

J.R. McLean
Bureau De La Radioprotection
Min. De La Santé Nationale Et Du Bien-Être Social
Ottawa, Ontario

L'étain est un métal lourd qui s'accumule dans le milieu par suite de l'activité industrielle. Au cours des années 1900-1972, sa concentration s'est accrue de 10 à 20 fois, l'augmentation la plus importante s'étant opérée entre 1962 et 1972, période précédant l'époque du fluorure d'étain dans les pâtes dentifrices et du chlorure d'étain dans les produits radiopharmaceutiques. Cependant, nos connaissances sont très limitées en ce qui concerne la toxicité infra-clinique de l'étain et en particulier de l'étain (II), sujet de cette enquête.

L'étain s'accumule rapidement dans les tissus à la suite d'une exposition et le corps le retient pour une longue vie biologique. Une analyse des cellules sanguines traitées au Sn(II) et Sn(IV) à l'aide d'un rayon de électrons indique que seul le Sn(II) pénètre tous les types de cellules étudiées et se répartit uniformément dans le cyptoplasme et le noyau. Dans certaines cellules, on peut découvrir des particules de Sn(II) de grosseur colloidale dans le cytoplasme.

Une injection d'une dose de <7 mg Kg^{-1} de chlorure d'étain (II) administrée à des souris, paralyse la synthèse ADN dans les cellules de la rate et de la moelle des os et élimine les réactions primaires d'immunité. On peut déceler des dommages ADN dans les cellules de la rate des souris que l'on a traitées in vivo au moyen d'une dose intra-péritonéale d'étain (II) de 7 mg Kg^{-1}, jusqu'à 24 heures après l'administration de la dose. Des études in vitro utilisant FADU et ASG attestent que l'étain (II) est dommageable au ADN des cellules HPBL et CHO. Le traitement du HPBL au moyen de 10 µM d'étain (II) entraîne le même nombre de lésions ADN, mesurées en brisures totales de chromosomes, qu'une exposition de 0.3 Gy aux rayons gamma Cobalt-60. On obtient plus efficacement des brisures de chromosomes ADN avec de l'étain (II) MDP et CDTA qu'avec du chlorure d'étain (II). La présence de 50 µM d'étain (II) dans le milieu d'incubation nuit dans une proportion de 75% à la réparation des brisures de chromosomes dans le HPBL.

Le chlorure d'étain (II) entraîne une suppression rapide et prolongée de la synthèse ADN dans les lymphocytes stimulés par la conconavalin A, ainsi que dans la ligne de cellule lymphoide transformée. Il suffit d'un traitement rapide des lymphocytes de souris ou d'humains au moyen de 25 µM d'étain (II) pour paralyser la synthèse ADN à 50%, mais seul 1 µM est nécessaire pour paralyser à 50% la ligne de cellule transformée. On considère que l'étain (II) affecte ainsi les cellules en occasionnant des dommages ADN qui entraînent la paralysie de la synthèse ADN in vitro et in vivo.

ACKNOWLEDGEMENTS.

I would like to express my gratitude to Dr.J.G.Kaplan for his guidance throughout this project. I would like to thank Drs. M.McBurney, H.C.Birnboim and G.C.Douglas for their support and helpful discussions, Dr.R.Pontefract for assistance with the microbeam analysis, Dr.D.Blakey for cooperation and patience with the alkaline sucrose gradients, Mr.J.Jevcak for his help with the mechanics of the FADU technique and Mr.L.Rockwell for his able technical assistance with the tissue distribution studies. To Mr.W.Welsh, Drs. P.J.Waight and E.G.Letourneau of the Radiation Protection Bureau for their patience and support, my colleagues in Professor Kaplan's laboratory for their advice and assistance and finally to my wife and family for their tolerance, and my daughter, Karen, for her valuable assistance in the completion of this work.

TABLE OF CONTENTS

	Page
Table of Contents	iv
List of Tables	x
List of Figures	xii
List of Photographs	xiii
Equations	
1. The Problem: The Approach	1
1.1 The Problem	1
1.2 The Approach	3
2.0 Introduction	4
2.1 Exposure to Chemicals	5
2.2 The Long Term Effects from Exposure to Metals	5
2.3 The Chemistry of Tin	8
2.3.1 Tin Bonding	9
2.3.1.1 The Chemistry of Stannous Chloride Dihydrate ($SnCl_2$)	10
2.3.2 Tin Complexes	11
2.3.3 Oxidation - Reduction Reactions	13
2.3.4 Radicals and Trace Metals	16
2.3.5 Environmental Chemistry: The Tin Geocycle	17
2.4 The Biology of Tin - Toxicology	19
2.4.1 Structure and Function of the Reticuloendothelial System	21
2.4.2 The Cellular Components of the Immune Response	22

		Page
2.4.3	The Phenomenon of Immunosuppression	26
2.4.4	The Somatic Mutation Theory and Long Term Health Effects - Mutation and Carcinogensis	28
2.4.5	An Alternate Hypothesis to Somatic Mutation	31
2.4.6	DNA Damage at the Molecular Level	33
2.4.7	Classes of DNA Lesions	34
2.4.8	DNA Damage Recognition and Repair in Eucaryotes	36
2.4.9	DNA Repair Pathways - Strand Break Repair	37
2.4.10	Self Destruction, DNA Disassembly or DNA Damage?	40
2.4.11	Prereplication Excision Repair	42
2.4.12	Postreplication Repair	43
2.4.12.1	DNA Replication on Damaged Templates	44
2.4.13	Inducible, Error-Prone Repair Synthesis	45
2.4.14	Consequence of Unexcised DNA Damage	47
2.4.15	Photoreactivation	47
2.5	Uptake, Distribution and Excretion of Tin by Mammals	48
2.5.1	Effect of Tin(II) on the Tissue Distribution of Tc-99m-Sodium Pertechnetate.	51
2.6	Metals and the Long Term Effects on Health; Carcinogenesis and Immunosuppression	53
2.7	DNA Synthesis Inhibition; A simple test for Agents that Damage DNA	57
2.7.1	Methods of Detecting DNA Damage	57
2.8	Alkaline Sucrose Gradient Analysis	59
2.9	The Fluorescence Analysis of DNA Unwinding (FADU)	60

	Page
3.0 Materials and Methods	62
3.1 Preparations of Solutions	62
3.1.1 Heavy Metals	62
3.1.2 Tin(II) Chelates	62
3.2 Cell Preparation and Culture Techniques	64
3.2.1 Mouse Cells	64
3.2.2 Human White Blood Cells	64
3.2.3 Human Peripheral Blood Lymphocytes (HPBLs)	65
3.2.4 Culture Techniques	65
3.2.5 Balanced Salt Solution (BSS)	66
3.3.0 Assessment of Uptake and Incorporation of Radionuclides into Cells	66
3.3.1 The Incorporation of Tritium (^{3}H) Labelled Macromolecular Precursors into Cells	66
3.3.2 The Uptake of Tin(II)-113 Into Cells in Culture	67
3.4.0 The Subcellular Distribution of Tin in Human WBC and Mouse Splenocytes	69
3.4.1 The Subcellular Distribution of Tin(II)-113 in Mouse Splenocytes	69
3.4.2 The Subcellular Distribution of Tin(II) and Tin(IV) by the Microbeam Analysis of Whole Cells	70
3.5.0 DNA Synthesis Inhibition Assays	71
3.5.1 The Effect of Tin(II) Treatment on the Ability of Resting HPBLs and Mouse Splenocytes to Respond to Conconavalin A	71
3.5.2 The Effect of Tin(II) Treatment on Transformed and Con A Stimulated Cells, In Vitro	72
3.6.0 Fluorescence Analysis of DNA Unwinding (FADU) for the Detection of DNA Strand Break Damage	73
3.6.1 The Direct Effect of Tin(II) on Isolated DNA	74
3.6.2 DNA Disassembly	75

Page

3.6.3 DNA Damage from Secondary Mechanisms: The Effect of the "Oxidative Burst" 75

3.6.4 The Effect of Tin(II) and Tin(IV) on Damage and Repair of DNA of Human WBCs at 0° and 37°C 76

3.6.5 The Combined Effect of Tin(II) and Cobalt-60 Gamma Rays on DNA Damage in Human WBC 78

3.6.6 The Detection of DNA Damage in the Organs of Mice by FADU 78

3.7.0 Alkaline Sucrose Gradient Analysis of DNA Damage in Chinese Hamster Ovary Cells 79

3.8.0 Tissue Distribution and Retention Parameters of Tin(II) in Mice 80

3.8.1 Tissue Distribution of Tin(II)-113 in Mice 81

3.9.0 The Effect of Tin(II) on the Tissue Distribution of ^{99m}Tc-Pertechnetate 82

3.10.0 Immunological Assays 82

3.10.1 The Assay for the Primary Immune Response-Plaque Forming Cells (PFC) 82

3.10.2 The Assay for one-way Mixed Lymphocyte Reaction (MLR) 84

3.10.3 The Effect of Tin(II) Treatment of DNA and Protein Synthesis in the Bone Marrow and Spleen Cells of Mice 84

3.11.0 The Statistical Analysis of the Data 85

4.0 Results 86

4.1 The Uptake of ^{113}Tin(II) by Cells in Vitro 86

4.1.2 The Effect of Chelating Agents on the Uptake of ^{113}Tin(II) by Human WBC In Vitro 91

4.1.3 The Subcellular Distribution of Tin(II) and Tin(IV) 94

4.1.3.1 Subcellular Fractionation of ^{113}Tin(II) 94

4.1.3.2 X-ray Microbeam Analysis of Human Peripheral White Blood Cells 96

Page

4.2 The Cellular Uptake of Tin(II) and the Inhibition of DNA Synthesis In Vitro 101

4.2.1. The Effect of Tin(II) and Tin(IV) on DNA Synthesis in Lymphoid Cells Stimulated by Con A 103

4.2.2. The Effect of Tin(II) on DNA Synthesis in Transformed Cells 105

4.2.3. The Effect of Tin(II) on DNA Synthesis in Human Raji Cells and Mouse EL-4 Cells 107

4.2.4. Tin(II)-113 Uptake and Inhibition of DNA Synthesis in Mouse Splenocytes Stimulated for 48 Hours with Con A 113

4.3.0. The Dection of DNA Damage In Vitro by Fluorescence Analysis of DNA Unwinding 114

4.3.1. The Detection of DNA Damage in Cells Treated with Tin(II) at 0° and 37°C 114

4.3.2. The Detection of DNA Damage in Whole Cells Treated with Tin(II) Chelates at 0°C and 37°C 118

4.3.3. How Does Tin(II) Damage DNA? Primary and Secondary Mechanisms 123

4.3.3.1 The Dection of Direct DNA Damage by Tin(II) in Cell Lysates 123

4.3.3.2 DNA Disassembly: The Effect of Cell Death on the Observation of DNA Strand Breaks by FADU 124

4.3.3.3 The Effect of 2-Deoxyglucose on DNA Damage from Treatment of Cells with Tin (II) Colloids 124

4.3.4. The Effect of EDTA on DNA Damage Produced in Human WBC by Treatment with Tin(II) 125

4.3.5. DNA Damage Produced in Human WBC from Combined Treatment with Tin(II) and Co-60 Gamma Rays 127

4.3.6. Validation of the Fluorescence Analysis of DNA Unwinding Validation of Technique In Vitro 127

4.3.7. The Effect of Tin(II) and Tin(IV) Treatment on the DNA of Chinese Hamster Ovary Cells as Analyzed on Alkaline Sucrose Gradients 131

4.4.0. The Effect of Tin(II) on the Immune System of Mice 133

		Page
4.4.1	The Effect of Tin(II) on the Primary Immune Response In Vivo	137
4.4.2	The Effect of Tin(II) on Mixed Lymphocyte Reaction (MLR)	139
4.4.3	The Effect of Tin(II) on Incorporation of ^{3}H-Thymidine into Spleen and Bone Marrow Cells In Vivo	139
4.5.0	The Biokinetics of Tin(II) In Mice	142
4.5.1	The Clearance Half-Times and Retention Parameters of ^{113}Tin(II) in Black Mice	147
4.5.2	The Tissue Distribution of ^{113}Tin(II) in Mice	147
4.5.3	The Metabolic Fate of Tin(II) In Vivo	151
4.5.4	The Detection of DNA Damage In Vivo by the FADU Technique	155
5.0	Discussion and Conclusions	158
5.1	The Uptake of Tin(II) by Cells	158
5.2	The Effect of Tin(II) Uptake on DNA Damage In Vitro	160
5.3	The Effect of Tin(II) on DNA Synthesis	164
5.4	The Effect of Tin(II) In Vivo	165
5.5	The Dose and Risk Estimates in Humans from Environmental Exposure to Tin(II)	171
5.6	Future Directions	173

LIST OF TABLES

		Page
Table 1.	Electronic Characteristics of Tin	9
Table 2.	Binding Constants of Tin	12
Table 3.	Redox Potentials of pH 7.0	15
Table 4a and 4b.	Deposition of Tin in Tissues	50
Table 5.	The Distribution of Tc-99m in Rats	52
Table 6.	The Uptake of ^{113}Tin(II) by Human White Blood Cells in Balanced Salt Solution at 0°C and 37°C	87
Table 7.	The Rates of Uptake (K) into Human White Blood Cells and the Clearance Half-Times (T) of ^{113}Tin(II) from Balanced Salt Solution at 0°C and 37°C	89
Table 8.	The Effect of Incubation Temperature on the Uptake of Tin(II) Chelates by Human White Blood Cells Suspended in Balanced Salt Solution	92
Table 9.	Detection of Tin in Human White Blood Cells by Microbeam Analysis	100
Table 10.	The Effect of Tin(II) Treatment on DNA Synthesis in Human Lymphocytes after Exposure to Con A for 96 Hours	106
Table 11.	Binding of ^{113}Tin(II) to Mouse El-4 Cells at Various Times After Treatment	110
Table 12.	Damage and Repair in the DNA of Human White Blood Cells Treated for 30 Minutes with Tin(II) at 0°C and 37°C	116
Table 13.	Comparison of Damage Produced in the DNA of Human White Blood Cells Exposed to Co-60 Gamma Rays and Tin(II)	117
Table 14.	Gy-Equivalent DNA Damage in Human WBC Treated by Tin(II) Chelates at 37°C., as Measured by FADU	119
Table 15.	Gy-Equivalent DNA Damage in Human WBC Treated by Tin(II) Chelates at 0°C, as measured by FADU	120
Table 16.	The Effect of Tin(II), Tin(IV) and Mercury(II) on cell Viability and FADU Results	122

Page

Table 17. The Effect of 2-Deoxyglucose (2-Dog) on DNA Damage in Human WBC Treated with Tin(II) Colloid Preparations 126

Table 18. The Effect of Tin(II) Treatment and Cobalt-60 Gamma Radiation on DNA Damage in Human White Blood Cells 128

Table 19. Damage to DNA, Measured by FADU, from Exposure of Human WBC to Various Agents 129

Table 20. Damage to DNA from Exposure of Human WBC to Various Carcinogenic and Non-Carcinogenic Metals 130

Table 21. The Detection of DNA Strand Breaks by Alkaline Sucrose Gradient Analysis in Chinese Hamster Ovary Cells Cells Treated with Tin(II), Tin(IV) and Chromium (VI) 132

Table 22. The Effect of Tin(II) on the Production of Plaque-Forming Cells (PFC) in Mouse Spleens 138

Table 23. The Inhibition of one-way mixed Lympocyte Reaction (MLR) in Murine Splenocytes Pretreated In Vivo for 2 Days with Tin(II) 140

Table 24. The Effect of Tin(II) on the Incorporation of ^{3}H-Thymidine into the Bond Marrow and Spleen Cells of Mice which have been Treated with Sheep Red Blood Cell (SRBC) Antigen 141

Table 25. The Tissue Distribution of ^{113}Tin(II) After Intravenous Injection into Mice 148

Table 26. The Tissue Distribution of ^{113}Tin(II) After Intraperitoneal Injection into Mice 149

Table 27. The Distribution of ^{99m}Tc-Pertechnetate in Mice 96 Hours After the Administration of Tin(II) at 7 $MGKG^{-1}$ by Intraperitoneal Injection 153

Table 28. The Effect of Orally Administered Tin(II) on the Tissue Distribution of 99m TC(VII) 154

Table 29. Intregrated Concentration of Tin(II) at end of D_n days 169

Table 30. Integrated Dose Expressed as Total Damage (Strand Break-Days/Genome) 170

LIST OF FIGURES

		Page
Figure 1.	The uptake of ^{113}Tin(II) by white blood cells in balanced salt solution at 37°C.	88
Figure 2.	Saturable Tin(II) uptake in human white blood cells at 37°C.	90
Figure 3.	The effects of Tin(II) (O) and Tin(IV) (●) on DNA synthesis in ConA stimulated mouse spleen cells.	105
Figure 4.	Incorporation of ^{3}H-thymidine into Raji cells exposed one hour to various concentrations of Tin(II).	108
Figure 5.	The rate of DNA synthesis in Tin(II) treated mouse EL-4 cells.	109
Figure 6.	Incorporation of ^{3}H-thymidine into Raji cells exposed for one hour to hydroxyurea and methylmethane sulfonate.	109a
Figure 7.	The dose-effect of Tin(II) on the rate of DNA synthesis in mouse EL-4 cells at 3 and 24 hours after treatment.	111
Figure 8.	The effect of Tin(IV) on Chinese hamster ovary cells.	134
Figure 9.	The effect of Tin(II) on Chinese hamster ovary cells.	135
Figure 10.	The effect of Chromium(VI) on Chinese hamster ovary cells.	136
Figure 11.	The retention of ^{113}Tin(II) in mice.	143
Figure 12.	The excretion of injected ^{113}Tin(II) from mice.	144
Figure 13.	The transfer of injected ^{113}Tin(II) from soft tissue to skeleton of mice.	150
Figure 14.	DNA damage in mouse splenocytes exposed in Tin(II) *in vivo*.	157

LIST OF PHOTOGRAPHS.

		Page
PHOTOGRAPH 1A.	A Mononuclear Leucocyte Ingesting a Particle of tin(II).	97
PHOTOGRAPH 1B.	Spectrum of Radioactivity from the characteristic x-Rays of Tin Accumulated, from the Nucleus	97
PHOTOGRAPH 2A.	A Human PMNL Ingesting Particles of Tin(II).	98
PHOTOGRAPH 2B.	Spectrum of Accumulated Radioactivity in Nucleus.	98
PHOTOGRAPH 3A.	A Human PMNL Ingesting a Variety of Tin Particles	99
PHOTOGRAPH 3B.	Spectrum of Accumulated Radioactivity in Nucleus.	99
PHOTOGRAPH 4.	A Large Accumulation of Tin(II) in a Human Leucocyte.	99

LIST OF EQUATIONS.

EQUATION 1.	The Clearance of Tin(II)-113 from Mice	145
EQUATION 2.	The Cumulative Concentration of Tin(II) in Tissues.	146

1. THE PROBLEM: THE APPROACH.

1.1 The Problem.

The problem to be investigated in this study can be stated, in general terms as follows: What are the mechanisms underlying the long term effects on health from exposure of organisms to toxic metals? This question is of genuine concern because metals accumulate in the environment as a result of industrial activity and therefore the level of exposure of the population to these substances can be controlled by political action. The biological consequences from chronic exposure to metals cannot yet be predicted from first principles because the relationship between the physical and chemical properties of the elements and their biological activities has not been established (Luckey, 1977). Therefore, each metal must be assessed individually for toxic properties. The long term consequences of chronic exposure to tin, a reactive group IVA element, is largely unknown because its importance as a pollutant in the ecosystem is a recent event (Hodge et al,1979).

A number of my observations, to be presented in detail in this thesis, indicate that exposure to tin(II),(Sn(II)), may represent a long term hazard to health, especially if the exposure is chronic. These observations, which are the basis for the formulation of a series of testable hypotheses, are summarized as follows:

1) Treatment of resting or stimulated cells with tin(II), but not with tin(IV), produces a dose-dependent inhibition of DNA synthesis and cell division *in vitro*.

2) Pretreatment of mice with intraperitoneal injections of tin(II) inhibits; DNA

synthesis and antibody cell formation in the spleen and bone marrow of mice that have been exposed to SRBC antigen.

3.Tin(II), but not tin(IV) induces DNA damage in Chinese hamster ovary (CHO) cells and human white blood cells (WBC) in vitro and in the spleen cells of mice in vivo.

4.The DNA damage produced from the simultaneous exposeure of cells to tin(II) and Co-60 gamma irradiation, is synergistic in that the damage from the two agents together is greater than the sum of damages from each agent alone.

5.The strand break damage in DNA produced by brief treatment with tin(II) is rapidly repaired but the effect on DNA synthesis is prolonged.

6. DNA that is released directly from cells by gentle lysis can be damaged by treatment with tin(II).

7. Tin(II) retains its activity in vivo for a prolonged period of time.

The general hypothesis can be stated as follows: Tin(II), but not tin(IV), is an immunosuppressive agent that exerts its effect by inhibiting the ability of lymphocytes to undergo blast transformation in response to antigens or polyvalent mitogens. This effect is due to a prolonged inhibition of DNA synthesis caused by DNA damage which can occur from in vivo or in vitro exposure to Sn(II). The Sn(II), inhibits repair in addition to causing direct DNA damage, is also a radiosensitizer and potentiates the damage caused by exposure of cells to ionizing radiation.

1.2 THE APPROACH.

The problem was approached in a logical sequence beginning with experiments designed to describe and characterize the biological effects of tin(II) on resting and stimulated lymphocytes and on various lymphoid cell types in culture. The uptake and subcellulor distribution of tin(II) by cells *in vitro* was characterized and the tissue distribution and retention parameters of ^{113}tin(II) injected into mice was described. The effect of tin(II) injection on the immune response *in vivo* on the ability of bone marrow and spleen cells to incorporate ^{3}H-TdR and on the capacity of splenocytes to act as responders and stimulators in one-way MLRs were investigated. A battery of short term assays to detect DNA damage from tin(II) were performed including, DNA synthesis inhibition, alkaline sucrose gradient analysis in CHO cells and FADU analysis in mouse and human cells. Finally, preliminary work was initiated involving, SCE analysis in tin(II) treated CHO cells, assessment of spermhead morphology in tin(II) treated mice and *in vivo* experiments designed to show that tin(II) remains active in the organs of mice days after it is administered by oral or parenteral routes.

2.0 INTRODUCTION

THE USES AND DISTRIBUTION OF TIN IN THE ENVIRONMENT.

Tin is present in the Earth's crust at 0.004% $_{w}^{w}$ and in sea water at a concentration of 0.003 g/ton (Bailar, 1973). Primary tin consumption in the industrialized western world was in excess of 120,000 tons in 1973 with about 75% of this being consumed by the food canning industry and 13% being converted to numerous organic forms for use in a variety of industrial processes such as, stabilizers for plastic films and polyvinyl chlorides and in fungicides, insecticides, anti-fouling marine paints and disinfectants (Bailar, 1973). More recently tin(IV) compounds have been tested as experimental anti-tumor agents (Crowe et al,1980). Stannous fluoride (SnF_2) is widely used as an anticaries agent in some toothpastes while stannous chloride ($SnCl_2$) is used as a preservative in some foods and beverages and as a reducing agent in a group of radiopharmaceuticals that are administered intravenously to patients undergoing diagnostic nuclear medicine procedures. The use and application of tin has rapidly expanded in society and its accumulation as a pollutant of the environment has doubled over the last 2 decades (Hodge et al, 1979). The insidious nature of heavy metal toxicity is well known and therefore, it would be relevant to explore the possibility that adverse effects on long-term health and well-being could result from chronic exposure of populations to tin(II).

2.1 EXPOSURE TO CHEMICALS;

THE PROBLEM OF LONG-TERM HEALTH EFFECTS.

There were 4 million distinct chemicals in man's environment in 1977 with an average discovery rate of about 6000 per week (Maugh, 1978). At least 63,000 chemicals are in common use today and others coming into common use at the rate of about 1000 per year. A small segment of the population is occupationally exposed to some 25,000 industrial chemicals. They have provided valuable information on the risk to health that is associated with high levels of exposure to some chemicals (Loprieno, 1977). The effect on health associated with exposure of a large heterogeneous segment of the population to a single chemical entity however is much more difficult to evaluate because exposure usually involves very low doses extending throughout a long-life span.

2.2 THE LONG TERM EFFECTS FROM EXPOSURE TO METALS.

There are 30 chemicals, including 4 metals nickel (Ni), chromium (Cr), arsenic (As) and beryllium (Be), established as human carcinogens (Maugh, 1978). The long-range effects on health that are associated with these types of metals is related to the loss in the fidelity of cellular information that is transferred during replication, transcription and translation. Immunosuppressive and mutagenic processes can develop in the cells of organisms as these information transfer processes deteriorate with the result that the onset, incidence and intensity of age-related diseases can be increased.

Metal ion cofactors are essential for the stability and fidelity of

information transfer in cells (Eichhorn, 1979). With increasing age these essential elements, which do not accumulate in the organism, have to compete with an ever increasing concentration of toxic heavy metals for binding sites on macromolecules. Some metals can degrade the ability of cells to process and transfer genetic information by affecting the integrity of the DNA template. They increase the mutagenic potential of the information transfer process by altering substrate and^{or} enzyme conformation and template-base specificity or by introducing crosslinking or bulky lesions into DNA. (Sirover and Loeb 1976), . (Clark and Eichhorn, 1974)(Eichhorn et al, 1979)(Sunderman, 1978)(Furst, 1977). Some metals can form redox couples that can catalyze the formation of DNA-damaging free radicals (Bisby et al, 1978) (Binder, 1979) or selectively degrade DNA and RNA by a nonenzymatic, hydrolytic mechanism (Butzow and Eichhorn 1965) (Butzow et al,1975)(Rosenthal et al,1966). The rate at which the information transfer process is degraded may be partly influenced by the rate at which heavy metals concentrate in the tissues. Generally, metals accumulate in the organism at a rate that is proportional to the concentration of the metal in the environment. Most toxic metals are colloidal at the pH of body fluids and therefore tend to accumulate initially in the phagocytic cells of the RES before being slowly transferred to the skeleton (Schroeder et al.1964). The strong affinity of the cells of the lymphoid tissues for metals ,implies that the functional capacity of the immune system can be compromised as metals accumulate. The development of an immune response requires an intact DNA template from which information can be constantly transcribed into the ribonucleic acids and the proteins that are necessary to maintain the capacity for clonal expansion in resting cells and for transformation of resting cells, after exposure to antigen, into functional blasts. If treatment of the lymphocyte with heavy metals can inhibit the process of blastogenesis then a direct and quantitative effect on the immune response can

be predicted.

Long term exposure of humans to compounds of As, Cr, Ni and Be can lead to an increased incidence of cancer and other age associated disorders. Induction of these disorders could be related, in part, to a progressive loss in function of the immune system and to the accumulation of DNA damage that could increase the chance of a cell expressing a mutated or transformed phenotype.

It is believed that the aging process is genetically determined which implies that there is age related changes in the structure of chromatin and DNA that could effect the ability of DNA to function as the repository of genetic information. These deleterious changes, in part, could be related to the accumulation of heavy metals in the cell. Several observations support these contentions:

(1) the body burden of heavy metals tends to increase with age and with the level of exposure (Bryan et al, 1976) (von Hahn et al, 1970);

(2) the ratio of essential to non-essential metal ions in chromatin changes with age (von Hahn et al, 1969) (Clark and Eichhorn, 1977) (Crapper et al, 1976);

(3) the exposure to toxic metals can lead to a variety of deleterious changes in chromatin and nucleic acid structure and function that can effect the transfer of genetic information (von Hahn et al, 1970) (von Hahn et al, 1969);

(4) There is a general decline with age, in the health and well being of the

exposed individual.

Two predictions can be made about the long-term risks associated with exposure of organisms to these types of metals. The metal could have an immunosuppressive effect or act as a mutagen or carcinogen with concomitant generalized aging effects on somatic cells. The remainder of this introductory section will discuss various aspects of these observations and speculations in greater depth.

2.3 THE CHEMISTRY OF TIN

Tin along with carbon, silicon, germanium and lead, occupy group IVA of the Periodic table of elements. All have electronic ground state configurations of $ns^2\ np^2$ (Hutchinson, 1959), with s representing sigma bonding orbitals and p the pi obitals and n the principal quantum number (Quagliano, 1969). The ground state configuration for tin is $5s^2 5p^2$. Like carbon, the bonding electrons of tin can be hybridized or mixed to give sp^3 or sp^2 orbitals (Abel, 1974) which can then form chemical bonds with strong covalent character. The preferred geometry of many tin(II) compounds is trigonal pyramidal or tetragonal pyramidal with the stereochemically active lone pair of $5s^2$ electrons occupying a position directed away from the strongly bonded coordination sites (Abel, 1978). The bond angles are $91\text{-}96^0$ for most tin compounds with bond lengths, depending on the bonded atom, in the order of 1.9-3.0 A (Abel, 1978).

Dilute acids attack tin metal to yield tin(II) salts and hydrogen gas (Abel, 1974). Tin(IV) salts hydrolyze to give insoluble oxides which are amphoteric.

Some physical characteristics of the tin atoms are summarized in table 1.

TABLE 1

ELECTRONIC CHARACTERISTICS OF TIN ATOMS.

Chemical Form	Ionization Potential, eV	Ionic Radii, A	Ground State
Sn(0)			$5s^2 5p^2$
Sn(II)	14.6	1.02	$5s^2 5p^0$
Sn(III)	30.7	-	-
Sn(IV)	46.4	0.71	$5s^0 5p^0$

2.3.1 TIN BONDING

Tin(II) is classified as a borderline acid in the hard- soft acid-base (HSAB) classification scheme of metals and forms predominantly covalent compounds. In contrast, tin(IV) is highly ionic in character (Nieboer et al, 1980). Metals can be separated into 2 distinct groups, based on empirical thermodynamic data, that is, in the trends in the magnitude of equilibrium constants that describe the formation of metal-ion/ligand complexes. On this basis, metal ions are separated into class A or hard acids and class B or soft acids (Nieboer et al, 1980) (Pearson, 1953) (Pearson, 1968a) (Pearson, 1968b) (Pearson, 1969). Class A metals or hard acids have considerable ionic character and prefer to react with ligands according to the following atom preference sequence: F Cl Br I and for metal-binding donor atoms in the sequence:

O > S = Se

N > As

O > N> S predominantly

In contrast, class B or soft acids have a predominantly covalent character and exhibit the opposite binding preference (Nieboer et al, 1980) (Arland et al,

1958). Class A metals then tend to be small, have a high charge/mass ratio and are not polarizable while class B metals tend to be large, with a low C/M ratio and are polarizable.

A third class of metal ions, referred to as borderline, (Nieboer et al, 1980) are less well defined but in general have a universal affinity for the above metal-binding electron donor atoms and ligands. In some cases the ligand preference is controlled kinetically, that is the binding of the ligand depends on the rate of the competing reaction of hydrolysis (Thomson et al,1972)(Chu and Tobias,1976).The hydrogen ion concentration (pH) is also an important factor in regulating the access of metals to binding sites in biological molecules since the proton, a borderline acid, directly competes with metal ions for these sites.

Stannous, Sn(II), is classified as a borderline acid with strong tendencies towards class B character. Stannic, Sn(IV), is also a borderline ion, but with strong tendencies towards class A character (Abel 1974).

2.3.1.1 THE CHEMISTRY OF STANNOUS CHLORIDE DIHYDRATE ($SnCl_2.2H_2O$.

In crystalline $SnCl_2.2H_2O$, one molecule of H_2O is coordinated to the SnCl molecule while the other is loosely bound as water of crystalization (Abel, 1974). In aqueous solution, in the presence of excess chloride (Cl^-) water can be displaced from its bonding orbital by the Cl^- and the Cl^- becomes coordinated into the vacated p orbital to form $SnCl_3^-$ (Abel, 1974). The tin(II) atom therefore is a p acceptor ligand. In addition, the directional lone pair of $5s^2$ electrons allow tin(II) dihalide to act as a sigma donor to metals such as platinium(Pt), iridium (Ir), ruthenium (Ru), rhenium (Rh) and iron (Fe) (Abel, 1974) (Binder, 1979).

Rinder has shown that tin(II) and Fe(III) can combine to form a redox couple in this manner. In addition to the strong sigma tin(II) metal bonds, the vacant p orbital of $SnCl_2$ can accept electrons from the filled d-orbitals of transition metals to further strengthen the Sn-metal bonding (Abel, 1978). The group IVA stannous dihalides (F^- and Cl^-) with a $5s^2$ outer electron configuration and an available p-orbital are called sigma-donor, p-acceptor ligands and are isoelectronic with carbene, carbon monoxide and ethylisocyanide (Dahl and Hodgson,1977). The carbon monoxide, Sn(II) dihalides and ethylisocyanide can complex with the iron of hemoproteins, such as cytochrome p-450 and give similar absorption spectra indicating similar bonding capabilities (Dahl and Hodgson,1977). Tin(II) has been shown to effect heme metabolism and will be discussed in a subsequent section.

2.3.2 TIN COMPLEXES.

Tin(II) binds to a wide variety of compounds as shown in table 2.

Table 2.

Binding Constants of Sn(II) and Various Compounds (Sillen, 1964, 1974)

Ligand	Log Binding Constant
F^-	6.3
Cl^-.	1.5
Phosphate (PO_4)	3.0
Pyrophosphate	4.5
Triphosphate	7.3
EDTA	22.0
CDTA	22.0

Tin(II) binds very strongly to F^-, pyrophosphate, EDTA and CDTA and weakly to phosphate, and by analogy to phytate (inosine hexaphosphate) and to chloride and citrate, although data is not available for the latter.

Tin(II) forms stable dioxytin compounds and can polymerize to form intermolecular tin-oxygen bridges (Abel, 1978). Tin(II) as the oxide crystals, binds to 4 oxygens to give a distorted square pyramid with Sn(II) at the apex and the lone pair of $5s^2$ electrons directed away from the planar face (Abel, 1974). Tin(II) also forms stable carboxylates and salts with oxyacids such as dithionates, carbonates, arsenates, vanadates, tungstates, borates, nitrates and sulfates (Jelen and Lindquist, 1969). (Nardelli et al,1975) For molecules like pyrophosphate, all bonding orbitals in Sn(II) are fully occupied by the oxygens from 2 molecules of pyrophosphate: bonding is strong, see Table 2 and the Sn(II) is essentially unavailable to participate in other reactions. The phosphate

anion, like Cl^-, binds only weakly to Sn(II) and therefore can be displaced readily by other more strongly bonding groups. The bonding orbitals of Sn(II) are fully occupied in complexes with EDTA (Smith, 1961) (Langer and Bogucki, 1967) (van Remootere et aT,1971 a,b). There is no hydrolysis of the Sn(II) EDTA complex which is also the case for Sn(II) CDTA (Langer and Bogucki,1967). EDTA is not taken up by cells either in vitro or in vivo (Rahman, 1979). CDTA is structurally related to EDTA but with more lipophillic character from the cyclohexyl group. Binding to CDTA is restricted to 2 bonding orbitals of Sn(II) which leaves 2 orbitals in Sn(II) occupied by weakly bound Cl^-. This is analogous to cis-platinium and the experimental antitumor organotin compounds where 2 good leaving groups like Cl^- occupy orbitals that are in the cis position. (Roberts, 1979) (Cleare et al, 1978) (Crowe et al, 1980). Phytate, inositol hexaphosphate, binds to Sn(II) rather weakly, like phosphate, except that each phytate molecule should accomodate up to 3 Sn(II) atoms. SnF_2, which forms colloid particals 0.1-10 nm in diameter (Amersham, personal communication) would have 2 Sn(II) bonding orbitals available for reaction.

2.3.3 OXIDATION - REDUCTION REACTIONS.

The reduction-oxidation potential (E^o, volts) is a measure of the tendency of a reaction to proceed relative to the reference hydrogen half reaction;

$$2H^+ + 2e = H_2$$

which is assigned a value $E^o = 0.0$ V (Quagliano, 1969a). By convention, a more negative E^o means that a reacting couple has a higher affinity for electrons and will easily accept them thereby acting as a oxidant. Similarly, a couple with a more positive E^o has a lower affinity for electrons and will donate electrons thereby acting as a reducing agent. When the redox couple, or half-reaction, is

written as follows;

$$\text{oxidized form} + e^- \quad \text{reduced form}$$

(oxidant) (reductant)

E^o values can be assigned for standard conditions defined at 25°C, 1 atm., 1 molar ion concentration at pH 0.0. The magnitude of the E^o value represents the strength of the oxidizing or reducing capacity of the particular redox couple.

The standard redox potential, E^o, can be adjusted to reflect biological conditions of pH 7.0 and 37°C (Lehninger, 1974) and is denoted by $E^{o'}$. A number of these adjusted redox potentials is shown in Table 3. In summary a positive $E^{o'}$ value means that the half reaction will proceed to the right while a negative one means that the half reaction will proceed to the left. Predictions can then be made about how redox couples will interact. Couples with more positive $E^{o'}$ values determine the direction of the reaction and the nature of the reaction products and thus couples with more positive $E^{o'}$ values will be the electron donors. From table 2, the following reactions can theoretically occur.

(1) $Fe(III) + Sn(II) = Sn(III) + Fe(II)$

(2) $O_2 + Sn(II) = Sn(IV) + H_2O_2$

(3) $Sn(II) + H_2O_2 = OH + OH^- + H_2O$

Table 3.

Redox Potentials Adjusted to pH 7.0

Reductant	Oxidant	Electrons	$E^{o'}$ (volts)
.OH	H_2	1	-1.25
O^-	O_2	1	-0.98
acetaldehyde	acetate	2	-0.60
Ferredoxin(Re.	Ferrodoxin(Ox.)	1	-0.43
H_2	$2H^+$	2	-0.42
NADH + H^+	NAD^+	2	-0.32
NADPH + H^+U	NADP	2	-0.32
Lipoate (Re.)	Lipoate (Ox.)	2	-0.29
Sn(II)	Sn(IV)	2	-0.27
Glutathione(Re.)	Glutathione(Ox.)	2	-0.23
Lactate	Pyruvate	2	-0.19
Cytoch.b (Re.)	Cytoch.b (Ox)	1	0.00
Ascorbate	dehydroascorbate	2	0.01
Ubiquinone(Re.)	Ubiquinone(Ox.)	2	0.03
Cytoch. C (Re)	Cytoch. C (Ox)	1	0.26
H_2O_2	O_2	2	0.26
Ferrocyanide	Ferricyanide	1	
Fe(II)	Fe(III)	1	0.35

2.3.4 Radicals and Trace Metals.

Singlet oxygen, superoxide anion and hydropgen peroxide are all formed in biological systems (see Singh, 1978 for review) as a result of normal enzymatic reactions involved in one electron transfers and from exposure of the organism to high energy radiation, either from natural or man-made sources. Many of the radical species can be interconverted at a rate that is rapidly increased by the presence of metal ions such as Fe, Co, Cr and Ni (Michelson, 1977). Some 53 radical reactions of potential biological importance have been described (Singh, 1978) but only those reactions which may involve catalysis by metals will be discussed here, such as:

$$(1)\ Fe(II) + H_2O_2 \longrightarrow .OH + OH^- + Fe(III)$$

$$(2)\ Fe(II) + O_2 \longrightarrow Fe(III) + O_2^{\cdot-}$$

$$(3)\ Fe(II) + O_2^{\cdot-} \longrightarrow H_2O_2 + Fe(III)$$

Reaction (1) is the well characterized Fenton reaction which converts H_2O_2 to the more damaging .OH. This reaction can also occur with ions such as Co(II) and Ni(II) (Pryor, 1978). The reversibility of reaction (2) is inhibited by the presence of anions such as F^-, pyrophasphate or OH^-, which can stabilize the O_2 product (Michelson, 1977). The reactions also can occur for metals such as Co(II) and Ni(II) but with different kinetics (Michelson, 1977).

2.3.5 Environmental Chemistry: The Tin Geocycle

Most of the uses of tin by society, in foods and beverages as preservatives, in toothpastes, radiopharmaceuticals, paints and fungicides, are dispersive and therefore this element readily enters into the natural waters, sediments and the biota (Hodge et al., 1979). The inorganic and methylated tin content in the surface waters of Lake Michigan and San Diego Bay is 38 PPM and 0.8-2.1 PPM in the surface waters several miles off the coast of California (Hodge et al., 1979). In coastal algae, the tin content was 1-2 PPM dry weight indicating that tin does accumulate in the food chain (Hodge et al., 1979). In addition, tin in cores drilled from marine sediments dated at 1972-73 were 20 times that found in cores dated at 1900.

The existence of a tin cycle has been recently described and its importance in the ecosystem will eventually determine the relative impact of tin pollution on the health of the population (Ridley et al., 1977). Tin, in this respect, must be regarded as a very toxic, relatively accessible substance (Wood, 1974), that can move through the environment by way of the tin geocycle. Industrial activity adds new sources of tin to this cycle which then makes the element available to the food chain. The metabolic activity of microorganisms is an important determinant in the mobility of tin in the environment, because they can convert inorganic tin into organotin compounds which are more toxic to

higher organisms. An important contributor to the tin geocycle is from the metal of discarded cans which can be slowly converted into reactive tin(II) by the action of dilute aqueous acids (Abel, 1974). The metal ion can then be biomethylated in the environment by microorganisms. The methylcorrinoids are the methylating agents relevant for metal salts because they are the only agents capable of transferring methyl groups as carbanions and as methyl radicals (Ridley et al.,1977). The methylcorrinoids have been implicated in the biomethylation of tin (Huey, 1974) and metals such as lead (Pb), palladium (Pd),Pt, gold (Au), thallium (Tl) and metalloids such as arsenic (As), selenium (Se), tellurium (Te) and sulfur (S) (Ridley et al.1977)

The methyl transfer to Sn involves a reductive homolytic cleavage of Co-C bond of the methylcobalamin with the transfer of CH_3 resulting in a 1 electron oxidation to Sn(III). Sn(IV) does not react with methylcobalamin. Sn(II), in the presence of single electron oxidants such as Fe(III) or Co(III), reacts according to the following equations (Ridley et al., 1977) (Dizikes et al., 1978):

$$Sn\ (0) + H^+ \cdot \quad Sn(II)$$

$$Sn(II) + Fe(III) \quad .Sn(III) + Fe(II)$$

$$.Sn(III) + CH_3\text{-}\ Co(III) \quad SnCH_3 + .Co(II)$$

$$(CH_3)_3\text{-}Sn(IV)Cl \quad .CH_3 + .Sn(III)$$

The methyltin compounds are relatively stable in aqueous solution but can be degraded by light to give the highly reactive stannyl radical.

2.4 THE BIOLOGY OF TIN - TOXICOLOGY.

The acute toxic effects of tin and its compounds have been studied but there is no recent reviews and no reviews at all dealing with the long term effects of tin. The following is a brief summary of the recent literature describing acute and chronic toxic effects from ingestion. or injection of inorganic tin(II).

Oral administration of tin(II) produced a dose dependent suppression of insulin secretion and hepatic phosphorylase activities in the rat (Yamaguchiet aT., 1978a,b). Tin treatment reduced glycogenolysis and glucose utilization in the brain (Cremer et al., 1970). Oral tin also inhibited duodenal active transport of calcium by inhibiting formation of the calcium binding protein and mucosal alkaline phosphatase activity. The biliary excretion of calcium increased and this together with the impaired uptake caused the serum concentration of calcium to fall (Yamaguichi et al., 1979) (Yamaguichi et al., 1978c). Tin, given by intraperitoneal or subcutaneous injection, decreased gastric secretion by inhibiting neuronal stimulation of the gastric mucosa (Yamaguichi et al., 1978d). Tin as Sn(II) depleted the brain and heart of catecholamines (More, 1961) (Robinson et al., 1969) and inhibited glutathione-s-aryltransferase in rats (Henry et al., 1976).

Stannous dihalides complexed with hemoproteins, such as cytochrome p.450, hemoglobin and peroxidase(Dahl and Hodgson,1978). Sn(II) ions directly affected the cellular content of heme proteins by inhibiting the production of aminolevulinate synthetase and stimulating the production of heme oxygenase, the rate limiting enzymes of heme synthesis and degradation (Kappas and Maines,1976)(Maines and Kappas,1977a).Tin also suppressed the cellular content of glutathione(Maines and Kappas,1977),a substance which is lost from tissues with age (Hazelton et al., 1980).Tin(II) suppressed the formation of 2 cytochrome p.450 dependent enzymes in mice (Burba and Rudd,1980). These actions of Sn(II) impair the oxidative function of cells thereby altering the impact of environmental chemicals which are detoxified by heme-containing microsomal systems (Maines, 1977b). Some recent studies indicate that tin may be an essential trace element for rodents (Schwarz, 1974). The relevance to human nutrition however is open to question (Nielsen et al., 1974).

2.4.1 Structure and Function of the Reticuloendothelial System

The reticuloendothelial system (RES) is a body of cells derived from the mesenchyme which form a diffuse system of sessile and mobile mononuclear macrophages. These cells have the common physiological property of being able to rapidly ingest and accumulate foreign colloidal and particulate matter (Saba, 1970).

The mobile macrophage component of the RES consists of blood monocytes and the wandering tissues macrophoges. tissues. The sessile macrophoges (stationary histiocytes), consist of the cells lining the sinuses of the lymph nodes and the liver, lung, spleen and bone marrow and the reticular cells of the spleen, central nervous system, lymph nodes and bone marrow. The macrophages of liver and spleen constitute 85-95% of the total intravascular phagocytic activity (Saba, 1970).

The fixed macrophages of the RES have been shown to be involved in the phagocytic clearance of colloidal and particulate matter, including autologous tissue debris antigens, denatured proteins and drugs.

2.4.2 The Cellular Components of the Immune Response.

Defective immune responses are known to be the result of primary immunodeficiency diseases such as thymic hypoplasia in man or in homozygous mice with the nude (nu^{nu}) mutation. Immune deficiency can also be secondary as a result of undernutrition, irradiation with x or gamma rays, neonatal thymectomy, treatment with certain drugs and chemicals, such as alkylating agents, and during infectious diseases (Boch et al., 1975) (Vos, 1977).

There are 2 distinct, but interdependent, systems of specific immunity in mammals based on experimental and clinical observation, see Greaves, 1973 and Vos, 1977 for more detailed discussions. Cell mediated immunity consists of those reactions which are operated and transferred by specifically sensitized lymphocytes. It includes the cell mediated, protective immunity against fungal, viral and bacterial infections and the rejection of allografts such as tumors and foreign tissue implants. The second immune system, humoral immunity, operates by antibody producing cells and is transferred by serum and includes the classical antibody mediated protective immunity which combats some bacterial and viral infections and anaphylactic reactions.

Lymphocytes play a central role in both types of immune response. They can be divided basically into B and T cells which have different functions and properties. The precursor cells of

lymphocytes, the hemopoietic stem cells, migrate in the embryo from the yolk sac through the liver to the marrow. Cells that are to become lymphocytes mature outside of the bone marrow. Some enter the thymus where they proliferate and mature under the influence of the thymic epithelial cells. A population of these cells are subsequently released from the thymus and become T-cells. The T-cell can circulate through the body and become temporarily resident in the thymus-dependent areas in the white pulp of the spleen (periarteriolar lymphocyte sheaths) and the paracortical areas in the lymph nodes. During stimulation, antigen is first processed by macrophages (Nossal et al., 1966) and then presented to the T-cells some of which are stimulated to divide in the process of blastogenesis (see Rosenstreich and Mizel,1978 for a comprehensive review of cell interactions). The clonal expansion of a few antigen specific cells produces a pool of sensitized T-lymphocytes, which can function as memory, effector, killer, helper and suppressor cells. Effector cells produce lymphokines (MIF, CAF, MAF) that can modulate macrophage function: killer cells are cytotoxic and have a key role in the rejection of tumors. Helper T cells collaborate with B-cells in antibody production against antigen. Suppressor cells suppress B-cells or effector T-cells.

The bursa of Fabricius in birds or the bursa equivalent in mammals, possibly gut associated lymphoid tissues or bone marrow, act as the central lymphoid organ in which stem cells are induced to become B-cells. B-cells circulate through the body and find

temporary residence in the thymus independent areas of the spleen (follicles and peripheral regions of the white pulp) and the lymph nodes (follicles and medulla). Upon antigen stimulation, B-cells specific for antigen, proliferate and develop into plasma cells that secrete antibody or serve as memory cells. IgM is produced early in the response to infection while IgG and the other classes of antibodies are synthesized primarily during the secondary response. For most antigens, cooperation between B and T-cells are necessary for the generation of an antibody response. This is referred to as a T-cell dependent humoral response. The cooperation of T-helper cells is not necessary for all antigens: E. Coli lipopolysaccharide is a thymus independent antigen.

Fixed and free macrophoges and their precursors, the promonocyte in bone marrow and monocytes in the blood, comprize a group of cells that are related by morphology, origin and function (Saba, 1970) (Langevoort et al., 1970). Macrophages are involved in antigen processing in which the antigen is degraded inside the cell, with some of the material being retained on its surface. The surface bound antigen and a soluble factor from the macrophage, interact with the lymphocyte to induce blastogenesis. This process generates a pool of sensitized lymphocytes as a result of the proliferation of precursor cells which begins 18-32 hours after antigen presentation (Dutton and Mishell, 1967). A similar lag period is found when mouse splenocytes are stimulated by the addition of SRBC to the culture medium (Mishel and Dutton, 1967). All the antibody forming cells arise by cell division with

different populations of cells being involved in a concurrent response to two non-cross-reacting antigens (Dutton, 1967) (Mishel and Dutton, 1967). This was shown by the use of a "hot" pulse technique where cells stimulated by SRBC to undergo DNA synthesis, were incubated with large doses of high specific activity ^{3}H-Thymidine (TdR). The resulting radiation caused DNA damage and a rapid and prolonged inhibition of DNA synthesis even after the cells were transferred to fresh medium without ^{3}H-TdR. The prolonged inhibition of DNA synthesis prevents the subsequent appearance of plaque forming cells. The effect of the hot pulse on PFC formation could be reversed if, at the time of addition to culture, the hot pulse was diluted with sufficient "cold" TdR to reduce the specific activity and to induce a reversible metabolic TdR block of DNA synthesis instead of an essentially irreversible block that would be produced by DNA damage. This type of experiment, in addition to showing that PFC's arise from clonal expansion of stimulated lymphocytes, also suggests that the immunosuppressive effect of whole body irradiation could be partly due to DNA damage in antigen stimulated cells.

2.4.3 The Phenomenon of Immunosuppression.

There is a wide variety of environmental, physiological and pathological conditions that can influence the immune system (Hudson et al., 1974). The hormones of the adenohypophysis exert a direct and indirect effect on the structure and functioning of the lymphoid organs and on the phagocytic activity of macrophages (Vos, 1977). Endogenous or exogenous adrenal glucocorticoids hormones induce lymphopenia and deplete the lymphoid tissues of cells, especially in the thymus. Glucocorticoids also cause sequestration of circulating monocytes and impair the production and release of monocytes which result in a reduced number of macrophages (van Furth et al.,1974). Glucocorticoids impair the uptake of antigen by macrophages and antigen recognition. Some drugs, such as oxisuran and cholera toxin, induce the production of glucocorticoids such as oxisuran and cholera toxin and these hormones are the actual immunosuppressive agents (van Dijk et al, 1975) (Morse et al., 1975).

Poor nutrition is known to induce increased production of glucocorticoids and ~~this~~ can lead to lympholysis and immune suppression. It becomes important therefore when studying the effect of environmental chemicals or drugs to determine if immunosuppression is accompanied by general atrophy and loss in body weight. Weight loss, and general atrophy may be related to the dose of chemical employed which may impair appetite,

absorption, transport and utilization of nutrients or the excretion of wastes.

2.4.4 THE SOMATIC MUTATION THEORY AND LONG TERM HEALTH EFFECTS - MUTATION AND CARCINOGENSIS.

The basic idea that somatic hereditary changes lead to malignant growth was put forward by Boveri (1914) and Tyzzer (1916). The concept that lesions in DNA are the causal factors in carcinogenesis has received much support in recent years but is still not universally accepted (Rubin, 1980).

Today, carcinogenesis is considered to be a multistep process in which a predisposing genetic constitution and changes to the genetic material from environmental factors play a dominant role in tumor development. The process has two major steps: initiation of a premalignant cell line and promotion of malignant growth. The initiation step most likely involves alteration of cellular DNA in the form of a permanent change or somatic mutation. Evidence implicating DNA damage in carcinogenesis is deduced from several lines of evidence:

(1) Known chemical carcinogens and ionizing or UV radiation cause lesions in DNA (San and Stich, 1975);

(2) The potential for tumor formation induced by UV irradiation, can be reversed by photoenzymatic repair of the resulting DNA damage (Hart et al, 1977);

(3) Many ultimate carcinogens are mutagens in the presence of metabolic activating systems (Huberman and Sachs, 1974);

(4) Individuals with rare genetic disorders involving a deficiency in DNA repair, have a high incidence of cancer. Fibroblast cell cultures from these individuals show deficient repair and have a high frequency of chromosome aberrations (Cleaver, 1968) (Wade et al., 1977) (Paterson, 1976);

(5) An increased cancer incidence from epidemiological studies correlates with a high frequency of chromosomal aberrations (Hemminki, 1979);

(6) There is an inverse relationship between

removal of specific DNA carcinogen adducts from specific tissues and the potential of a carcinogen to induce tumors in that tissue (Goth, and Rajewsky, 1974) (Lewis and Swenberg, 1980);

(7) Neoplastic transformation has been induced experimentally in hamster cells treated sequentially with bromodeoxyuridine(BudR) and near UV radiation which directly perturbs DNA and results in chromosome damage (Barrett et al., 1978);

(8) Tumors are mostly monoclonal in origin, implying that a genetic change in one cell leads to tumor growth;

(9) "Cured" cancer patients have an increased risk of a second malignancy by enhancement of new somatic mutations through radiation and chemotherapy (Nichols et al., 1975).

2.4.5 An Alternate Hypothesis to Somatic Mutation.

There is however some arguments against the theory that somatic mutation or damage to DNA is the causal event in the transformation of cells to the malignant state. The current impetus of the somatic mutation theory comes from the observation of Ames (Ames et al., 1973) that many chemical carcinogens can produce mutations in bacteria when the chemicals are metabolically activated. However, a number of noncarcinogens have produced mutations in the Ames test and some of the most powerful carcinogens are only weakly mutagenic (Rubin, 1976) (Sivak et al., 1976) (Simmon et al., 1979). In addition, in the phenomenon of surface carcinogenesis (the Oppenheimer effect), tumors can be induced by inserting solid sheets of chemically inert material into the tissues of rats (Alexander and Horning, 1958). No tumors are formed if the material is made fibrous or porous. The malignant transformation threfore seems to depend on the physical state of the material. There is no indication that the solid material gains entry to the cells. In this case it could be disruption of the organized behavior of cells that leads to the malignant transformation (Rubin, 1980).

Treatment with chemical carcinogens in vitro allow cells to escape from the density-dependent regulation of growth. When these cells are injected into an appropriate host they retain their aberrant growth behavior and form tumors. Such a phenomenon is termed malignant transformation. The frequency of transformation has been found repeatedly to be on inverse function of the cell population density at the time of treatment (Reznikoff et al., 1973). At very low concentrations all cells are transformed by the chemical treatment without any cell death and therefore there is no selection of transformed cells (Mondal et al., 1970). In this case, the transformation frequency appears to depend on

the number of times, after exposure to a chemical carcinogen, that a cell can be kept dividing before coming into contact with other cells in the colony. The frequency of chemical transformation at a relatively low cell density has been estimated to be 10^{10} times higher in culture than in situ in the animal (Parodi and Brombella, 1977). The importance of cellular interactions then could be the critical parameter in the regulation of growth and metabolism. Most nonexcitable normal cells have been found to be electrically coupled to one another and ions and other small molecules can flow freely from cell to cell through gap junctions (Loewenstein et al., 1966) indicating that there is a high degree of metabolic cooperation among normal cells. Many tumor cells however, are electrically uncoupled and are, in effect, isolated from the influence of surrounding cells (Simpson et al., 1977).

The initiating events in cell transformation could occur at the membrane of the cell according to Rubin (1980). In this model the carcinogen destabilizes the membrane which initiates changes leading to the irreversible loss in the capacity of the cell to interact with other cells. The probability of malignant transformation occurring would increase. There is no requirement for the carcinogen to interact directly with DNA (Rubin, 1980). However, it can also be argued that the carcinogen, interacting with the DNA, could bring about changes in the expression of genetic information that could affect the structure of the membrane so that the cell would become uncoupled from the control of surrounding cells. This could then lead to malignant transformation.

Throughout the thesis, the tenets of the somatic mutation theory, that DNA is the primary target for carcinogens, will be accepted because of the strength of the supporting evidence. It follows then that the accumulation of DNA damage

is the basis for many adverse long term changes in the health status of the individuals.

2.4.6 DNA DAMAGE AT THE MOLECULAR LEVEL.

Damage to various components of cells exposed to certain chemical and physical agents can lead to impairment of biological function. Chromosomal DNA is a particularly sensitive target for the action of these agents because its high molecular weight makes it fragile, and its chemical properties make it reactive. Genomic information may be affected by DNA damaging agents at the molecular and chromosomal levels. Lesions in DNA can be expressed at the molecular level as point mutations (base-pair substitutions or frameshift changes) or at the chromosomal level where structural changes (deletions, rearrangement and breaks) or numerical changes (aneuploidy and polyploidy) can occur.

2.4.7 CLASSES OF DNA LESIONS.

Several types of DNA lesions can be recognized:

1. Overt breaks, which can be single or double stranded;

2. Alkali-labile lesions that are converted to single strand breaks by alkali treatment, such as base damage, base-free apurinic/apyrimidinic (AP) sites or phosphotriesters;

3. DNA-DNA interstrand or intrastrand crosslinks;

4. DNA - protein crosslinks.

The extent and type of distortion in the DNA helix and the alteration in base pairing and stacking capacity, depends on the type of lesion (Grossman et al., 1975) (Kohn et al., 1979). The extent and type of helix distortion in turn may select the type of repair pathway that is used to restore the integrity of template. This may largely determine how the biological effects of such damage are expressed at the cellular level (Cerutti, 1975).

The structure of chromatin, consisting of DNA wrapped around histones to form the nucleosome, which are then then organized into higher orders of coiling, probably plays a dominant role in the interactions between DNA and chemical carcinogens and in control of the sites and rates of excision repair (Metzger et

al., 1977). Large carcinogen adducts, DNA-DNA and DNA-protein crosslinks may have restricted locations and are nonrandomly distributed in chromatin (Cleaver, 1978). In contrast, the strand breaks from exposure of cells to x-rays and pyrimidine dimers from UV, have approximately a random or uniform distributions in DNA.

2.4.8 DNA DAMAGE RECOGNITION AND REPAIR IN EUCARYOTES.

There are 4 general categories of DNA repair in eucaryote cells:

1. Strand break repair;

2. Prereplicative repair;

3. Postreplication repair;

4. Photoreactivation repair.

DNA repair is a dynamic process in which various repair mechanisms or pathways coexist. The position of the cell in the cell cycle and the state of differentiation in the tissue may also be factors in determining which repair pathway is dominant. In addition, there is always competition between error-free and error-prone pathways for repair of DNA damage and this may determine the mutation load that is carried by the cell (Verly et al., 1980). Two mechanisms leading to mutations can be considered:

1. Minor base modifications leading to mispairing, and

2. Major DNA damages inducing error-prone "SOS" repair processes.

In both cases avoidance of mutation relies on prereplicative excision repair. The mutation frequency appears to depend on a competition between error-free

excision repair of damaged sites and DNA replication which can be either normal de novo synthesis or error-prone "SOS" DNA synthesis (Verly et al., 1980). If excision repair enzymes are able to excise and repair damage before normal de novo synthesis occurs, then the mutation frequency remains low. If normal DNA replication occurs before damage is repaired then mispairing can occur and a mutation can be fixed in the template. The "SOS" error-prone repair enzymes are induced by major DNA damages and the integrity of the DNA template is restored without regard to base fidelity with the result that the mutation rate is increased.

2.4.9 DNA REPAIR PATHWAYS - STRAND BREAK REPAIR.

Strand breaks may result from the presence of alkali-labile base damage or A.P. sites in DNA (Bertrom et al., 1974); alkali-labile phosphotriester groups in alkylated DNA (Walker and Ewart, 1973); cellular metabolism, so called DNA disassembly (Williams, 1974); the action of excision repair enzymes (Hart, 1978); and the direct action of chemical radicals produced by chemical reactions or physical agents.

Double strand breaks can arise from a single ionizing event that interacts simultaneously with both strands of DNA or by two independent interactions occurring in close proximity, in time and space, on complementary DNA strands.

The number of single strand breaks that are formed in mammalian cells that have been exposed to a damaging treatment, is independent of the position of the cell in the cycle (Ormerod, 1977). Sulfhydryl agents and free-radical scavengers in high concentration can reduce the number of radiation induced breaks.

Mammalian cells in culture, rapidly rejoin single strand breaks in DNA after exposure to ionizing radiation, with 50% being rejoined during the first 10 minutes of postirradiaion incubation and 90% being rejoined at 60 minutes (Ormerod, 1977). The rate of strand rejoining is energy dependent and can be inhibited at temperatures of 4°C, by a lack of ATP, by incubation with 2,4 dinitrophenol and other inhibitors of oxidative phosphorylation, and by anoxic incubation conditions (Ormerod, 1976). Rejoining of single strand breaks occurs maximally at 40°C for human lymphocytes and is independent of RNA; protein and DNA synthesis (Ormerod, 1976). The rejoining of single strand breaks occurs in all normal resting cells, cells undergoing division or cells that have been stimulated to undergo division. The enzymes necessary to rejoin strand breaks therefore appear to be a normal component of the nucleus and may be a part of the enzyme system normally involved in DNA replication or in maintaining the integrity and fidelity of the DNA sequences. Rejoining of single strand breaks appears to occur without any distrurbance of the genetic code (Humphrey et al., 1968).

In contrast, the restitution process for double strand breaks is less efficient than that for single strand breaks (Resnick et al., 1976) but repair does occur with increased incubation times (Corry and Cole, 1973). The restitution process for double strand breaks could occur with the loss or addition of nucleotide base pairs to the chain so that the restoration of mechanical integrity could be coupled with disturbances in the genetic code which could lead to a somatic mutation (Hart, 1978). Double strand breaks have been proposed as the rare event that gives rise to chromosome aberrations and cell death Leenhouts and Chadwick, 1978.

Some data indicate that agents such as cystamine and 2 mercaptoethylamine (MEA) reduce the number of single and double strand breaks that occur in x-irradiated fiberoblast cultures. This results in an increase in survival fraction (Ormerod, 1971) suggesting that these lesions are the lethal events induced by both ionizing radiation and selected chemical carcinogens. However, these sulfhydryl reagents also protect DNA against other forms of damage induced by ionizing radiation and hence such data can only suggest, indirectly, a link between the induction of strand breaks and biological processes such as lethality, mutagenicity, and transformation (Hart, 1979).

A correlation does exist between the capacity of 4NQO derivatives to induce DNA strand breaks at protein linker sites and carcinogenicity of these agents Andoh and Ide, 1972. The induction of strand breaks and oncogenic transformation with SV-40 virus has also been reported (Brakeslee et al., 1976).

2.4.10 SELF DESTRUCTION, DNA DISASSEMBLY OR DNA DAMAGE?

It is important to prove that the DNA degradation, observed by FADU or ASG analysis, in cells treated with Sn(II), is not simply DNA disassembly due to cell death or to some non-DNA damaging trauma. It must be shown that Sn(II), or some radical derived from an interaction with Sn(II), produces a direct damage to the DNA of cells.

Physical and chemical DNA damaging agents produce a spectrum of damages in the DNA of exposed cells. Some of this damage is expressed as strand breaks or as strand breaks after exposure to alkali. In contrast, cells that have been exposed to a variety of traumas, including radiation, can undergo a sudden endonucleolytic reduction in DNA size (Williams et al., 1974). This reduction in size can occur within minutes of the trauma or after several hours (Lett et al, 1972) (Shipley et al., 1971) or after progression of cells into the late S-G_2 phase of the cell cycle (Williams et al., 1976). The reduction in size of DNA is under cellular metabolic control and results primarily from an intrinsic enzymatic attack on DNA.

The process of DNA disassembly to 50S fragments can be triggered by agents that damage DNA only or induce damage at the cell membrane or produce general cellular trauma (Williams et al., 1976). If the damage to the cell is extensive then DNA disassembly continues to 5S fragments and may precede or occur concommitantly with cell death. If the cell is capable of surviving the level of damage inflicted by the agent, then the DNA will be restituted and the cell will recover. The time of initiation of DNA disassembly can be extended in heavily traumatized cells by holding them at conditions which do not permit cell

metabolism (Williams et al., 1976). The molecular signal to initiate disassembly appears to be a nuclear one and requires an energy source because isolated nuclei, with stable, intact DNA, initiate disassembly with the addition of only ATP to the incubation mixture. This demonstrates that the source of the endonuclease is not the lysosomes and that DNA disassembly is an energy dependent process that appears to be under nuclear control. The disassembly process may share initial pathways with repair enzymes since it could be a means of selecting for death those cells that are heavily damaged, while allowing those cells with less severe damage to survive and proliferate.

The possibility that colloidal Sn(II) particles can cause DNA damage indirectly by triggering the release of H_2O_2 from the granulocytes in the mixed white cell population, must also be entertained. The H_2O_2 released after phagocytic stimulus or after treatment by membrane active agents, diffuses into the extracellular medium and is capable of producing damage in the surrounding cells(Hafeman and Lucan,1979)(Clark et al., 1979). In addition, the H_2O_2 can be converted to the more potentially damaging .OH radical by trace metals (Tauber et al., 1978). This respiratory "burst" of oxidizing activity is accompanied by a consumption of glucose. The respiratory burst can be blocked by treating the cells in the presence of 2-deoxy-glucose, a non-metabolizable analog of glucose (Kay et al., 1980).

2.4.11 PREREPLICATION EXCISION REPAIR.

Prereplicative excision repair is a complex set of enzyme pathways with various degrees of specificity for damaged sites. In its simplest form, excision repair involves the recognition of damage, the removal of damaged parental DNA and its replacement by DNA that is synthesized using the opposite strand as template. In one repair pathway, nucleotide excision, the damage is recognized by a repair endonuclease which then excises the entire nucleotide. In base excision, an N-glycosylase removes the damaged base by the hydrolysis of the N-glycosidic bond, leaving the phosphodiester backbone intact. This action is then followed by apurinic or apyrimidinic endonucleolytic hydrolysis of the phosphodiester bond. Subsequent steps, which may be common to both pathways, involves action by an exonuclease, which removes nucleotides around the damaged site, a polymerase to replace the damaged region and a ligase to rejoin the strand segments. The repair pathway apparently is largely controlled by the nature of the lesion (Hart, 1978) (Friedberg et al., 1977) (Duker and Teebor, 1975).

Following damage recognition and incision by an endonuclease, or N-glycosylase, there is a degradation of the DNA strand by an exonuclease which results in the release of the damaged region including a variable number of other undamaged nucleotides. The number of nucleotides released depends upon the form of damage induced. In the case of UV-induced pyrimidine dimers, the patch size in DNA is about 80-100 bases (Painter et al., 1972) whereas for x-radiation induced lesions the patch size is 1-3 bases (Regan and Setlow, 1974). It has been suggested that base excision repair corresponds to small patch or x-ray-like repair and large patch or UV-like repair corresponds to nucleotide excision

repair (Cleaver, 1979).

The third step in the repair process involves the activity of a DNA repair polymerase. A mammalian DNA polymerase has been isolated with a 3' to 5' exonuclease activity (Byrnes et al., 1976), analogous to a number of prokaryotic DNA repair polymerases which behave like a proof-reader and ensures error-free DNA synthesis.

The final step in the repair process involves the sealing of the newly synthesized DNA to the parental DNA strand by the action of a polynucleotide ligase (Segev et al., 1973). This step in excision repair could lead to mutation by the resealing of nicked DNA before the damaged region has been removed.

2.4.12 POSTREPLICATION REPAIR.

Postreplication repair is the process by which the DNA replication machinery of the cell copes with lesions in the parental DNA strand. It is not strictly a repair process in mammalian cells because the damage is not removed. This concept was derived from the observation that newly synthesized DNA in UV irradiated cells was initially of lower molecular weight than in unirradiated cells (Rupp et al., 1968). The gradual increase in molecular weight on subsequent incubation in the postreplication period was interpretted as the repair of gaps formed in the daughter DNA opposite the lesion in the parental strand. This type of repair is important to cells that are actively synthesizing DNA or cells that are unable to excise all lesions from their DNA prior to the onset of DNA synthesis.

The introduction of damage into the DNA of cells can produce a rapid and prolonged inhibition in DNA synthesis. Damage to the DNA blocks chain growth causing the production of low molecular weight DNA. Replication then resumes beyond the damaged area, leaving a gap, which is later filled by de novo synthesis (Cleaver, 1979). Damaged sites are not removed from DNA during these molecular weight changes and instead of repair the process probably represents de novo DNA synthesis in the presence of perturbations in the DNA chain. The phenomena observed in damaged cells include: inhibition of average rates of DNA synthesis, inhibition of chain elongation and, possibly, the induction of an error-prone DNA repair enzyme (Sarasin et al., 1978), the so-called "SOS" repair pathway of Radman et al., 1975.

2.4.12.1 DNA REPLICATION ON DAMAGED TEMPLATES.

Domains of supercoiled DNA, about 10^9 daltons, appear to be the units of replication under common regulation (Hand et al., 1978) (Cleaver, 1979). Initiation of DNA replication at many origins is coordinately controlled within the domains, which in turn may be controlled by the higher orders of chromatin coiling. Replication occurs bidirectionally from many independent replicating forks at the rate of 0.5 - 2.5 um/minute/replicating fork, The distance between origins is in the order of 15 - 200 um (Hand et al., 1978). After replication, adjacent replicons are joined together to form the intact DNA strand.

Many agents can inhibit DNA replication, but one characteristic of DNA-damaging agents is that upon their removal from the incubation medium, DNA synthesis remains inhibited because the damage remains in the DNA with a half-life that depends on excision repair (Cleaver, 1979). Agents that inhibit

replication without damaging DNA do so only when they are continuously present; replication rapidly recovers when these agents are removed from the incubation medium (Painter, 1977) (Painter, 1978). This distinction is the basis of a rapid method for indentifying inhibitory agents that damage DNA and are potentially carcinogenic (Painter, 1977). Damage that leaves moderate to high cell survival causes long periods of low rates of DNA synthesis, paritcularly when there is damage on both strands ahead of the replicatidn fork (Cleaver, 1979). The low synthesis rates could be due to changes in the rates of chain growth, premature chain termination, inhibition of replicon initiation or combinations of these (Cleaver, 1979).

The dose-response relationship observed for rates of DNA synthesis in x-irradiated cells show a radiosensitive component that is inhibited strongly by doses of 10Gy and a radioresistant component inhibited at higher doses. The sensitive component is due to the inhibition of replicon initiation (Cleaver, 1979) and this occurs after exposure of cells to MMS or bromouracil photolysis. Calculations of the target size for x-ray induced damage suggests that one single-strand break within a domain of 10^9 daltons may be sufficient to prevent initiation at all sites throughout that region of DNA (Cleaver, 1979). These single-strand breaks probably alter the supercoiled configuration of DNA which could prevent replicon initiation, possibly by preventing the binding of initiation proteins.

2.4.13 INDUCIBLE, ERROR-PRONE REPAIR SYNTHESIS.

The presence of an inducible error-prone "SOS" repair process in eucaryotic cells, similar to that found in bacteria (Radman et al., 1975) has aroused

considerable interest. In bacteria, UV irradiation triggers mutagenesis, lysogenic induction, cell filamentation and phage reactivation (Witkin, 1976). The expression of these effects is closely associated with RecA and lexA gene functions. A general theory of inducible SOS repair (Sarasin et al., 1978) has been adapted from work on procaryotes, to explain the enhancement of postreplication survival and mutagenesis observed in eucaryotes after treatment with UV (d'Ambrosio and Setlow, 1976) and x-rays (Kennedy and Little, 1978). Briefly, a regulatory signal caused by lesions in DNA initiates derepression of functions leading to the induction of an error-prone DNA repair process. The exact biochemical nature of this inducing signal is not known but it could be the inhibition of DNA synthesis, DNA degradation or the release of degradation products such as nucleotide monophosphates. (Sarasin et al., 1978). In eucaryotes, UV irradiation blocks DNA synthesis and the size of the DNA synthesized after irradiation is comparable to the size of the interdimer spacings (Lehman et al., 1972), indicating that gaps exist in the DNA strands opposite the lesions in the parental strands. Gap filling by a DNA polymerase reconstitutes the integrity of the newly synthesized strand and enhance survival of the cells. The mutagenic potential however is increased if the gap filling is accomplished by an error-prone polymerase. The survival of mammalian cells is enhanced by incubation with an uncoupler of oxidative phosphorylation before, during or after x-irradiation (Laval et al., 1980). This enhanced survival is correlated to an increase in expression of a mutated phenotype suggesting that gap filling is accomplished by an error-prone enzyme system that was induced by the x-ray treatment (Laval et al., 1980). The increase in mutagenic expression is greatest in cells that are treated in plateau phase. Thus, postrepliation repair processes decrease cell lethality but also are a source of mutations.

2.4.14 CONSEQUENCE OF UNEXCISED DNA DAMAGE.

Cells from different tissues have different repair capacities for similar forms of DNA damage (O'Connor et al., 1973). Neural tissues remove N_7 ethylguanine or 3-ethyladenine adducts at the same rate as other tissues, but they remove O6 ethylguanine at a reduced rate (Magee et al., 1975). The phagocytic Kupffer cells from rat liver (Lewis et al., 1980) excise and repair O6 methylguanine damage more slowly than do hepatocytes. This leads to accumulation of DNA damage in the Kupffer cells after chronic exposure (Lewis and Swenberg, 1980) which eventually can lead to the induction of a variety of liver cancers. The reduced rate of removal of O_6 ethylguanine and other carcinogen adducts in different tissues may relate to differences in saturation levels of the enzymatic repair system or to the state of differentiation in the tissues. This provides a correlation between low levels of excision repair of some adducts and the induction of mutagenesis and carcinogenesis in some tissues after chronic exposure to DNA damaging agents.

2.4.15 PHOTOREACTIVATION.

The photoreactivation repair system for monomerizing UV induced cyclobutane-type pyrimidine dimers will not be discussed because it bears little relevance to this thesis. For recent reviews however see Hart (1978).

2.5 UPTAKE, DISTRIBUTION AND EXCRETION OF TIN BY MAMMALS.

Normal intake of tin in man is estimated to be 1.5 to 45 mg/day (Schroeder et al., 1964) (Hiles, 1974). About 2.9% of Sn(II) and 0.6% of Sn(IV) is absorbed by fasted rats after a single, acute, oral dose (Hiles, 1974)(Furchner and Drake, 1976) but 16-20% can be absorbed if the dose is administered in an acid vehicle (Hiles, 1974). Lower absorption rates occur during chronic administration of oral tin in the presence of food (Hiles, 1974).

The whole body retention of ^{113}tin(II) administered by injection is described by a 4 component exponential expression and is similar for mice, rats, dogs and monkeys (Furchner and Drake, 1976). After administration, 42% is excreted with a half-time of 0.8 days and 15%, 16% and 27% with half-time values of 5.2, 24 and 96 days respectively. This indicates that about 43% of the administered dose is cleared only slowly from the tissues (Furchner et al., 1976). Brown et al (1977) resolved the excretion curve into only one component with a clearance half-time of 29 days. A similar clearance curve was found for ^{113}Indium, a radioactive daughter of ^{113}tin (Brown et al.,1977). Injection of excess unlabelled Sn(II) into mice 6 to 8 days after dosing with ^{113}tin(II) did not change the excretion rate of radioactivity indicating that Sn(II) metabolism may not be under homeostatic control (Brown et al., 1977) (Schroeder et al., 1964) as is the case for many essential metals. Hiles, 1974, found that 47% of Sn(II) and 54% of Sn(IV) were retained by rats 48 hrs after parenteral administration, while after oral dosing 42% and 40% of Sn(II) and Sn(IV) respectively was retained although most of this was within the gastrointestinal tract.

^{113}Tin(II) and ^{113}tin(IV) have distinct tissue distribution patterns in

mammals and there is no interconversion between oxidation states during absorption and systemic transportation or after being fixed in the tissues of rats (Hiles, 1974). Tin(II) tends to accumulate initially in the liver, spleen and kidneys after systemic or oral dosing. Tin(IV) in contrast, concentrates in the kidneys reaching levels that are 20 times those found in the liver at 48 hrs (Hiles, 1974). Tin(II) and Sn(IV) are both initially fixed in the Kupffer cells of the liver (Khan et al., 1956) (Hiles, 1974) and are retained with little turnover. Systemic Sn(II), but not Sn(IV), from non-RES sources is partially excreted in the bile (Hiles, 1974). About 50% of the initial Sn(II) dose in the liver is eliminated after 140 days while tin in the testes and spleen remain relatively stable (Furchner et al., 1976). The concentration of Sn(II) in bone steadily increased with time while blood concentration remained uniformly low (Furchner and Drake, 1976). The half-times of Sn(II) and Sn(IV) in bones was estimated to be about 4 months (see Hiles, 1974, for a review). An accumulation of Sn(II) occurs in the small intestine with time and is possibly related to metabolic activity. From the literature, the distribution of Sn(II) and Sn(IV), after I.V. administration, can be contrasted as follows:

TABLE 4.A

THE DEPOSITION OF TIN(II) IN THE TISSUES OF MICE AFTER THE ADMINISTRATION OF A SINGLE ORAL 20MG KG^{-1} DOSE.

	% of a single 20mg kg^{-1} oral dose retained at 48 hours.	
	Sn(II)	Sn(IV)
Skeleton	1.02	0.24
Liver	0.08	0.02
Kidney	0.09	0.02

TABLE 4.B

THE DEPOSITION OF TIN IN THE TISSUES OF MICE AFTER THE ADMINISTRATION OF A SINGLE INTRAVENOUS DOSE OF 2 MG KG^{-1}.

	% of injected dose of 2mg kg^{-1} retained after 48 hours.	
	Sn(II)	Sn(IV)
Stomach	0.1	0.004
Small Intestine	0.5	0.04
Large Intestine	1.0	0.2

2.5.1. EFFECT OF TIN(II) ON THE TISSUE DISTRIBUTION OF Tc-99m-SODIUM PERTECHNETATE. ($^{99m}TcO_4$)

Stannous ion is widely used as a reducing agent in the preparation of ^{99m}Tc-labelled radiopharmaceuticals. The reaction, which is only poorly understood, involves the reduction of the chemically inert ^{99m}Tc(VII) by tin(II) to give a chemically reactive ^{99m}Tc(III) or Tc(IV) species which then can complex with a variety of ligands to form gamma emitting radiotracers (Lin et al., 1975) (Eckelman et al., 1975). The Sn(II) may or may not form an integral part of the radiopharmaceutical (Deutsh et al., 1976)(Burns and Marzilli, 1977).

The tissue distribution of ^{99m}Tc has been well characterized in humans and rodents (Ancri et al., 1977) (McRae et al., 1974) (Patel et al., 1979). The normal tissue distribution pattern of ^{99m}Tc-pertechnetate can be altered in rats (McRae et al.,1974) and humans (Ancri et al.,1977) that have received prior doses of Sn(II). Minimal, but statistically significant changes in tissue distribution have been noted with I.V. doses of Sn(II) as low as 0.02 mg/kg in the rat and changes in this distribution pattern could be detected 13 weeks after 8 mg of Sn(II)/kg. The shift in the distribution pattern of $^{99m}TcO_4$ occurred when Sn(II) was given as the chloride, gluconate or citrate (Khentigon et al., 1976). The ingestion of oral Sn(II) by rats, either as Sn(II)UD dissolved in water at 0.19 mg/ml or as Fresca, a soft drink (Coca Cola Company) which contains 0.25 mg^{ml}, resulted in a significant alteration in the normal *in vivo* distribution pattern of $^{99m}TcO_4$. In this case $^{99m}TcO_4$ was administered intravenously 12 days after the last dose of Sn(II) (Khentigan et al., 1976). The shift in $^{99m}TcO_4$ distribution by the prior administration of tin(II) is shown in table 5.

TABLE 5

THE DISTRIBUTION OF ^{99m}Tc IN RATS ONE WEEK AFTER RECEIVING A TIN(II) INJECTION AT 8 MG KG^{-1}.

% of Injected Dose of $^{99m}TcO_4$ in Rats 1 Week After Receiving Sn(II) at 8 mg^{kg}

	Control (-tin)	Tin Pretreatement
RBC	0.06	5.1
Serum	0.5	5.5
Liver	4.3	11.6
Kidney	1.0	2.9
Stomach	17.1	1.8
Femur	0.2	0.4
Skeleton	7.3	12.3
Skin	27.4	6.9

2.6 METALS AND THE LONG TERM EFFECTS ON HEALTH;CARCINOGENESIS. AND IMMUNOSUPPRESSION.

Nickel (Ni), chromium (Cr), arsenic (As) (Sunderman, 1978) and beryllium (Be) (Hassan and Kazemi,1974) are generally accepted as human carcinogens on the basis of epidemiological evidence. Ni, Cr, and Be cause tumors in experimental animals but As does not (Sunderman, 1978). Lead has not been shown to cause cancer in humans but it does cause kidney cancer in experimental animals after oral administration (IARC, 1972) and increased chromosomal aberrations have been found in lead exposed workers (Deknudt et al., 1973). All of the carcinogenic metals so far studied bind extensively to the components of chromatin (Reiner et al.,1971)(Costa, 1980)(Oskarsson et al., 1979)(Levis, 1978). Be, Ni and As have a high affinity for the chromosomal proteins. Be and Ni bind weakly to the phosphate backbone of DNA (Norseth, 1978). Cr(III), binds strongly to the phosphate backbone and irregularly to the DNA bases (Levis, 1979).

Be and Cr cause rapid and prolonged inhibition of DNA synthesis in normal and regenerating rat liver and in fibroblast cultures repectively (Norseth, 1978) (Levis, 1978). Ni produces a rapidly reversible inhibition of DNA synthesis that is characteristic of non-DNA damaging agents (Painter, 1977). In addition, soluble Ni salts are not mutagenic in the Ames test (Venitt et al., 1974) and do no cause extensive DNA damage in the FADU assay (McLean and Kaplan, 1981). These observations indicate that As, Ni, which associate with the chromosomal proteins and in particular with the non-histone proteins, may induce mutations by altering the pattern of gene expression (Costa, 1980) (Reiner et al., 1971). Cr(VI) is rapidly taken up by cells *in vivo* and *in vitro* while Cr(III) is largely excluded

(Levis, 1978), however, Cr(III) is 20 times more potent in producing base mispairing in cell-free systems (Sirover et al., 1976). Chromium, is probably taken up by cells as Cr(VI) and then quickly reduced to the more mutagenic Cr(III) (Costa, 1980). Water soluble metal compounds of Ni(II) and Cr may be weakly carcinogenic in vivo because they are rapidly excreted from the body by the kidneys and there is only minimal tissue contact time. Carcinogenicity can be increased if the metal is slowly released from a reservoir or depot such as occurs during the slow solubilization of relatively insoluble particles or from a metal prosthesis that is corroded by body fluids. In these cases there is a constant source of tissue exposure to the metal ions and the tissue contact time is extended. The extensive deposition of heavy metals in a particular tissue or cell type can induce cell death. This may be followed by differentiation and proliferation of adjacent cells in an attempt to restore cell number. Proliferation on damaged templates could then lead to neoplastic transformation.

As(III) at 50 um irreversibly inhibits DNA synthesis in CHO cells and lower concentrations cause x-ray-like division delay (Gurley et al., 1980). This delay is correlated with reduced phosphorylation of histone proteins but there is no evidence that this perturbation in chromatin metabolism can results in genetic damage, although derepression of oncogenes cannot be ruled out (Gurley et al., 1980).

Metals which are carcinogens in experimental animals have a variable effect on DNA synthesis in mitogen stimulated lymphocytes. Mercury as Hg(II), lead as Pb(II) and Cr(III) are mitogenic at highly cytoxic concentrations (Shenker, 1977 for review). Lymphocytes isolated from the spleens of mice treated orally with Hg(II), cadmium as Cd(II) and Pb(II) have a reduced response to stimulation by

PHA and PWM (Gaworski and Sharma, 1978). The treatment of lymphocytes, in vitro, with copper as Cu(II), cobalt as Co(II), manganese as Mn(II), nickel as Ni(II) and cadmium as Cd(II) are inhibitory to transformation by PHA (Berger et al., 1974), while Zn and Fe are stimulatory (Shenker, 1977) (Chesters, 1972). These results indicate that metals which have carcinogenic potential in animals may also be capable of inducing immunosuppression by virtue of their inhibitory effect on DNA synthesis. Immunosuppression can be primary in that there is an inhibition of PFC formation that is not due to selective cytotoxicity whereas secondary immunosuppression occurs when the treatment produces cell death (Vos, 1977). Some organotin compounds are selectively cytotoxic for the thymus and spleen (Seinen et al., 1979) whereas many inorganic compounds such as Cd(II) and Ni(II) are immunosuppresive because of their general cytotoxicity (Kutz et al., 1980). The inorganic salts of Pb, Cd and Hg have been reported to be immunosuppressive in vivo (Koller et al., 1973) as have the organic compounds of Pt (Berenbaum, 1971), Hg(II), Pb(IV) and As(III) (Blakey et al., 1980). Immunosuppression in the case of Pb was probably due to cytotoxicity (Vos, 1977). The relevance of this type of suppression is not known because cytotoxicity at the high doses used in the experimental procedure may not occur at the very low doses obtained during actual environmental exposure. However, very low doses, especially if cumulative may produce adverse effects on DNA (Lewis and Swenberg, 1980).

The spleen and thymus cells are extremely sensitive to radiation and DNA damaging drugs (Geraci et al., 1975) (Goh et al., 1976). After exposure of the thymus to therapeutic x-rays as infants, adults still have elevated levels of chromosome aberrations in peripheral blood, a lower number of circulating B and T cells, an inhibited response of peripheral blood lymphocytes to PHA and an

increased incidence of neoplasms (Goh et al., 1976). Therefore, a single large dose of a DNA damaging drug or chemical may produce an exaggerated effect in experimental animals; low but cumulative doses may also produce the same but in a more insidious manner (Campbell et al., 1980).

2.7 DNA SYNTHESIS INHIBITION; A SIMPLE TEST FOR AGENTS THAT DAMAGE DNA.

An early response of various mammalian cells to chemical carcinogens is an inhibition of DNA replicative synthesis. The carcinogens DMBA and dimethylnitrosamine, inhibit DNA replication in regenerating rat liver (Craddock, 1975) (Marquardt et al., 1972). Methylazoxymethanol acetate, which induces colon tumors, also inhibits DNA replicative synthesis in colon mucosa *in vivo* and *in vitro* (Mak et al., 1978) (Zedeck et al., 1977). The molar dose of eight N-nitroso compounds, which are inhibitory to DNA synthesis in rat esophogeal epithelium, correlates significantly with carcinogenic dose (Mirvisch, 1978). The inhibition of mouse testicular DNA synthesis by chemical carcinogens likewise shows a high correlation with carcinogenicity (Seiler et al., 1977). Covalent binding of carcinogens to cellular DNA, resulting in damage to DNA template, has been observed for many types of chemical carcinogens (Heidelberger et al., 1975). Many chemicals however inhibit replicative DNA synthesis by altering cell metabolism without binding to DNA thereby weakening the correlation between the ability of compounds to inhibit DNA synthesis and to produce DNA damage.

2.7.1 METHODS OF DETECTING DNA DAMAGE.

Painter (Painter, 1977) systematically documented the effects of known DNA damaging agents and nondamaging metabolic inhibitors on the rates of DNA synthesis in human HeLa cells *in vitro*. Treatment of cells with DNA damaging agents produced a rapid and prolonged inhibition of DNA synthesis while general metabolic inhibitors produced a reversible suppression of DNA synthesis. These two types of inhibitors could therefore be distinguished on the basis of the rates of change in DNA synthesis after removal of the agents from the incubation

medium.

The explanation for these observations is that when an agent that inhibits DNA synthesis by metabolic means, such as $NiCl_2$, DMSO, cycloheximide or hydroxyurea, is removed from the medium the metabolic block is removed and recovery begins immediately and the rate of DNA synthesis increases. However, when a DNA damaging agent, such as benzpyrene, MMS, or 4NQO, is removed from the medium the damage to DNA remains and the rate of DNA synthesis decreases with time. This also occurs for cells treated with UV light or chemicals that mimick the action of UV light. These treatments induce lesions in DNA that block the progression of growing points in replicating DNA until repair occurs. Agents, such as x-rays and MMS, block the initiation of replicons, so that DNA synthesis progressively decreases as more and more replicons terminate after damage is incurred. These results suggest that measurements of DNA synthesis within the first 1-2 hours after treatment with an agent can determine its potential as a mutagenic and probably carcinogenic agent (Painter, 1977).

Painter (Painter, 1977) has incorporated these observations into a procedure that is designed to rapidly screen compounds for DNA damaging properties. The test measures thymidine uptake into the DNA of human (HeLa) cells at various times after treatment with a presumptive carcinogen or mutagen. Agents, such as cyclophosphamide, that require prior metabolic activation can also be assayed by this procedure (Painter, 1977) (Painter, 1978).

The use of a repair deficient XP cell line instead of the HeLa cell did not increase the sensitivity of the test (Painter, 1978). In this assay system, $NiCl_2$ inhibited DNA synthesis by slowing but not blocking the rate of chain

elongation, in a manner similar to cycloheximide and hydroxyurea and therefore is presumed not to be DNA damaging at 500uM . Metallic, insoluble Ni compounds (Ni_3S_2)are known carcinogens in rodents and in humans (Oskarsson, 1979). Phorbol myristate acetate, a tumor promotor for mouse fibroblasts, also inhibited DNA synthesis, in a reversible manner like $NiCl_2$.Metabolic poisons, such as potassium cyanide (KCN), a respiratory inhibitor (Keilin, D, 1929) at 15-30 mM decreased DNA synthesis initially but recovery occurred quickly when the cells were transferred to fresh incubation medium (Painter, 1977). The inhibiting effect of hydroxyurea (HU), DMSO and ethanol on DNA synthesis was also rapidly reversible. HU inhibits ribonuoleotide reductase and decreases the intracellular pool size of deoxyribonucleosides while DMSO and ethanol are membrane active agents that alter nucleoside transport into cells(Adams and Lindsay,1967)(Plagemann and Richey,1974). This indicates that metabolic inhibitors, that decrease the size of deoxyribonucleoside pools or interfere with nucleoside transport, must be continuously present in the incubation medium in order to inhibit DNA synthesis. A similar correlation was found using an assay system consisting of mouse spleen cells activated in vitro by Con A for 48 hr before treatment with carcinogens (Warren and Summerville, 1980).

2.8 ALKALINE SUCROSE GRADIENT ANALYSIS.

In the usual procedure for alkaline sucrose gradient analysis, a small number of cells are lysed in an alkaline detergent solution (pH 12) that has been layered on top of a gradient of alkaline sucrose (McGrath et al.,1966) (Palcic and Skarsgard, 1972). Cell lysis, removal of associated molecules and denaturation of DNA by the alkali are accomplished on top of the sucrose gradient without any mechanical handling of the DNA. Centrifugation of this denatured DNA

at optimal conditions of rotor speed and tube dimensions results in a random molecular weight distribution on the gradient which sediments in the range of 120-180 S (Cleaver et al.,1975)(Lett et al.,1967). The assay conditions are adjusted so that the single strand molecular weight peak in untreated controls is reduced to about $5x10^8$D. either by careful selection of the alkaline lysis conditions (Lett et al., 1967) or by introducing a controlled number of breaks into DNA by exposure to x-rays (Lehmann and Ormerod, 1970). Reliable molecular weight distributions can be obtained when the single strand molecular weights of control DNA is reduced to 5×10^8D. This system can then be used to quantitate the damage introduced into the DNA of cells that have been exposed to various agents by comparing the change in the MW of the treated DNA to that of control DNA. Alkaline sucrose gradient analysis can be used to quantitate total strand breaks introduced into DNA directly as strand breaks or indirectly from alkali-labile lesions.

2.9 THE FLUORESCENCE ANALYSIS OF DNA UNWINDING (FADU). Method For Detecting DNA Strand Breaks.

An extensive discussion of the method has been presented by Birnboim (Birnboim and Jevak, 1981). Briefly, large double stranded DNA molecules are released from mammalian cells by gentle lysis. This duplex DNA slowly unwinds when exposed to alkali. The rate of this unwinding is increased when breaks are introduced into the DNA by treatment of whole cells with a DNA damaging agent. The increased rate of unwinding can then be used as a measure of the relative number of strand breaks produced in the DNA by the treatment. The degree of unwinding, or the amount of duplex DNA remaining, is determined fluorometrically by the use of ethidium bromide, a dye that binds selectively to duplex DNA. The

difference in fluorescence between a control sample and a treated sample is a measure of the damage produced by the treatment.

3.0 MATERIALS AND METHODS.

3.1 PREPARATIONS OF SOLUTIONS.

3.1.1 HEAVY METALS.

Stannous dichloride dihydrate crystals were purchased from several sources (Fisher, British Drug House, Merck and Anachemia). Clear crystals were weighed and dissolved immediately in 1-2 mls of M/1 HCl, passed through a 0.45 um polycarbonate filter, when necessary, and then brought to volume with sterile distilled and deionized water so that the final concentration of HCl was M/100. The tin(II) content was standardized by iodometric titration using M/10 or M/100 iodine(I_2) with starch as indicator, (USP XIX). All solutions were prepared fresh for each experiment and held at 0^o until used. Stannic chloride solutions were prepared in a similar manner.

All other heavy metals were obtained as reagent grade inorganic salts from Sigma Chemical, Fisher Scientific, Merck or Anachemia. The solutions were made as 100x aqueous concentrates and added to the cell suspensions to give final concentrations of 50 uM with respect to the metal ion.

3.1.2 TIN(II) CHELATES.

Tin(II) at 500 uM in M/1 HCl was reacted with aqueous solutions of methylene diphosphonate (MDP), pyrophosphate (PyP), human serm albumin (HSA), ethylenediaminetetraacetic acid (EDTA), trans-1, 2-diaminocyclohexane N,N,N'N' tetraacetic acid (CDTA), citrate, phosphate, phytate (inositol hexaphosphate) to form tin(II) chelates (Abel, 1978). Chemicals were purchased from Sigma or Aldrich Chemical. Tin(II) Fluoride colloid was obtained from Amersham. Several

of the above chelates are available commercially and these were also obtained and tested. Several preparations containing tin(II) complexed to denatured (aggregated) human serum albumin were obtained from NEN and C.E.Frosst for testing. The chelate was always in a 200 times molar excess of the tin(II) for all of the complexes.

^{113}Tin(II) Chloride was obtained from NEN at 18.2 mCi mg^{-1} in 4M HCl. Radiolabelling of the tin(II) chelates was accomplished by an exchange process whereby 0.056 uCi of ^{113}tin(II) was added to a solution of the nonradioactive tin(II) before being reacted with an excess of the chelate (Abel, 1974).

3.2 CELL PREPARATION AND CULTURE TECHNIQUES.

3.2.1 MOUSE CELLS.

Mice were sacrificed by cervical dislocation and their spleens quickly excised, pressed through a stainless steel screen and then suspended in ice cold complete medium (RPMI-1640 10% fetal calf serum (FCS) (Flow Labs.), 2 mM L-glutamine, pen-strep (100 iu and 100 ug per ml) and 50 uM 2-mercaptoethanol(2-ME). Clumps and debris was allowed to settle out and then the crude suspension was aspirated off and the cells collected by centrifugation (400 g, 5 min, $0^{o}C$). The cell pellet was resuspended in 0.87% ammonium chloride, 10 mM tris buffer (pH 7.2-7.4), and held at $0^{o}C$ for 10 minutes to lyse the red blood cells (RBC). Cells were collected by centrifugation, washed and then adjusted to give $2x10^{6}$ nucleated cells per ml. Viability, as determined by trypan blue exclusion, was always greater than 90%. Typically, 200 to $500x10^{6}$ cells were obtained from each spleen, consisting of about 40% large monocytes and multilobed granulocytes.

3.2.2 HUMAN WHITE BLOOD CELLS.

Total WBCs were isolated from human peripheral blood by diluting 1 volume of blood with 3 volumes of a RBC lysing solution, as described above, and holding the mixture at ice bath temperature for 20-25 minutes. Cells were collected at 260 G for 10-15 minutes at $0^{o}C$. The supernatant was aspirated off and the cells were washed once in fresh lysing solution and then resuspended in a modified Earle's salt solution (BSS) or medium as dictated by the treatment protocol.

3.2.3 HUMAN PERIPHERAL BLOOD LYMPHOCYTES (HPBLs).

Peripheral venous blood from healthy human donors was withdrawn into 60 ml syringes containing 2-3 ml of sodium heparin (100 I.U./ml). The contents of each syringe was mixed with 40 ml of RPMI-1640 culture medium and this diluted blood was gently layered onto 15 ml of Ficoll-Paque (Pharmacia) in 50 ml plastic tubes (Falcon). The tubes were centrifuged at 18°C for 30 minutes at 400 g. The lymphocyte layer was removed following centrifugation, washed twice in complete medium and cell number and viability were determined by trypan blue exclusion and hemocytometer.

3.2.4 CULTURE TECHNIQUES.

Mouse cells at $2x10^6$/ml were suspended in complete medium at pH 6.9 and incubated at 39°C. HPBL were suspended at 10^6/ml in complete medium at pH 7.3-7.4 and incubated at 37°C in an atmosphere of 5% CO_2. Wild type Chinese hamster ovary cells were cultured in minimal essential medium (Gibco) supplemented with 10% FCS, non-essential amino acids and sodium pyruvate in an atmosphere of 5% CO_2 and high relative humidity.

3.2.5 BALANCED SALT SOLUTION (BSS).

BSS was a modification of Earle's balanced salt solution (Birnboim et al., 1981) as is made as follows;

magnesium sulfate ($MgSo_4.7H_2O$)	0.100 g
potassium chloride (KCl)	0.400 g
sodium Chloride (NaCl)	8.000 g
Hepes Buffer	5.960 g
Dextrose	1.000 g
Deionized distilled water	1.0 L
pH at 7.2-7.4	

3.3.0 ASSESSMENT OF UPTAKE AND INCORPORATION OF RADIONUCLIDES INTO CELLS.

3.3.1 THE INCORPORATION OF TRITIUM (^3H) LABELLED MACROMOLECULAR PRECURSORS INTO CELLS

Stock solutions of ^3H-Thymidine (^3H-TdR), ^3H-Uridine (^3H-UdR) and ^3H-Leucine (^3H-Leu) were obtained from Amersham at 50 Ci/m mole. These stocks were diluted with RPMI-1640 to give secondary stock solutions of 10 or 100 uCi/ml for pulse labelling cells.

One ml aliquots of cells, appropriately labelled with the ^3H-precursor, were washed sequentially onto premoistened glass fiber filters (GF/c Whatman), under gentle vacuum, first with 25 mls of ice cold phosphate buffered saline (PBS),

25 mls of ice cold 5% trichloroacetic acid (TCA), and then with 30 mls of 95% ethanol. The radioactivity retained on the filter was assessed by liquid scintillation counting using 3-5 mls of Scintillene (Fisher Scientific).

3.3.2. THE UPTAKE OF Tin(II)-113 INTO CELLS IN CULTURE.

^{113}Tin(II) dichloride was purchased from New England Nuclear at a specific activity of 18.2 mCi/mg and a radionuclidic purity of 99%. ^{113}Tin decays 100% by electron capture with a half-life of 118 days to give ^{113}indium (In) which then decays by gamma emission at 393 KeV at a half-life of 100 minutes (CRC Handbook, 1969). The detection of the ^{113}tin is done by determining the decay of its ^{113}In daughter in a 2"x 2" sodium iodide gamma detector (Amersham AutoCounter). The ^{113}Tin(II) dichloride is extremely reactive in dilute solutions binding nonspecifically to glass and cellulose. All solutions were therefore manipulated in plastic or siliconized glass containers and pipettes. Wherever possible carrier tin(II) was added to reduce this nonspecific adsorption. ^{113}Tin(II) tends to rapidly form a radiocolloid in weakly acidic solutions (Bailer, 1974) and the particle size increases as the concentration of the carrier tin(II) increases (Nelp, 1969).

Polycarbonate was the only material which did not bind ^{113}tin(II) nonspecifically. Binding of ^{113}tin(II) to cells could be assessed by gently washing them through filter discs of polycarbonate (1 um pore size, Biorad Labs.) with 30 ml of ice cold PBS and then determining the gamma count at 393 KeV.

The preferred method however, was the rapid microfugation technique of Strauss et al., 1977). The assay was done in 0.4 ml microfuge tubes (Beckman) by layering 0.2 ml of cells, at $2x10^6$/ml, over a layer of silicone oil (Silicone 556, 13 parts and Silicone 550, 12 parts, Dow-Corning). The cells were centrifuged at 12,000 rpm for 15 seconds in a Beckman Microfuge (model B-9). The

tubes were quickly frozen in liquid nitrogen and the tips, containing the sedimented cells, were cut off and placed in gamma counting tubes. The spin through from control tubes (incubation medium and ^{113}tin(II) and no cells) was always less than 0.465% of the total radioactivity in the incubation medium.

^{113}Tin(II), diluted with the appropriate carrier, was added as a 25x to 100x concentrate to suspensions of mouse splenocytes or human WBC at $2x10^6$/ml. The pH of the cell suspension under these conditions remained in the range of 7.1 - 7.3. Cell suspensions were maintained in a constant temperature bath at 0°C or 37°C., with gentle mixing every 2-3 minutes. Triplicate 0.2 ml aliquots of cells were removed from the incubation mixture every 2.5 minutes and centrifuged through oil as previously described, to determine the time course of radionuclide uptake. In some experiments the concentration of the radionuclide was kept at a constant specific activity and the cell number was varied. In other experiments, whole blood was incubated with ^{113}tin(II) at 37°C to determine binding to RBC.

The uptake of ^{113}tin(II) chelates was done at a concentration of 50 uM tin(II) and 1 mM of the chelator. This resulted in an adequate level of carrier tin(II) that made manipulation of the solutions relatively easy. Human serum albumin (HSA) at 2% (about 200 uM) was mixed with an equal volume of ^{113}tin(II) with carrier, and 0.1 ml of this was added to every 0.9 ml of cell suspension, to give a final HSA concentration of 0.1% and tin(II) at 50 uM.

3.4.0. THE SUBCELLULAR DISTRIBUTION OF TIN IN HUMAN WBC AND MOUSE SPLENOCYTES.

3.4.1. THE SUBCELLULAR DISTRIBUTION OF Tin(II)-113 IN MOUSE SPLENOCYTES.

The procedure of subcellular fractionation used here is an adaptation from Mahler, 1966. Briefly, splenocytes from one mouse were isolated and suspended at $5x10^6$/ml for 30 minutes in BSS containing ^{113}tin(II) at 1 uCi/ml in the presence or absence of 10% FCS. At the end of the incubation period duplicate 0.2 ml aliquots were taken for 100% reference counts and duplicate 0.2 ml aliquots were collected on polycarbonate filters to determine the total cell bound radioactivity. The remaining cells were collected by centrifugation (250 g, 10 min, 0^oC), washed 3 times in ice cold PBS and adjusted to $20x10^6$/ml in lysis buffer. The lysis buffer consisted of 10 mM Hepes, 50 mM potassium chloride (KCl), 5 mM magnesium acetate and 0.05% triton-X 100 at pH 7.8. A counting reference of 0.2 ml of this cell suspension was taken in duplicate. At the end of 15 minutes incubation, the cells were observed by phase contrast microscopy to ensure that intact nuclei were present. The intact nuclei were collected by centrifugation (450 g for 10 min., 4^oC). The supernatant was removed and the nuclei gently resuspended in the same volume of fresh lysis buffer and incubated for a further 15 minutes. The intact nuclei were again collected and washed two more times in a solution consisting of 0.25 M sucrose and 0.003 M calcium chloride ($CaCl_2$). The pellet was gently resuspended in the same volume of buffer and the radioactivity for in 0.2 ml aliquots was compared to that found in the counting reference.

3.4.2. THE SUBCELLULAR DISTRIBUTION OF TIN(II) AND TIN(IV) BY THE MICROBEAM ANALYSIS OF WHOLE CELLS.

Human WBC were left untreated or incubated with tin(II) or tin(IV) in BSS for 15 minutes at 37°C. The cells were washed 3 times in ice cold BSS and then fixed for 1.5 hours in 2.5% glutaraldehyde, dehydrated through an ethanol series, transferred to propylene oxide and then embedded in Spurr as described in detail by Glauert, 1975). Embedded cells were sectioned, stained for 20 minutes each in uranyl acetate and then lead citrate. Serial sections were mounted onto gold grids to prevent interference with the detection of the tin K-alpha 1 and 2 characteristic x-ray peaks. Specific areas of the cytoplasm and the nucleus were irradiated with a 3 uAmp beam current 0.2 to 0.5 um in diameter and counts from the tin x-rays were collected for 300 seconds in a multichannel analyzer set so that each channel was 20 Kev in width. RBC, monocytes, lymphocytes and granulocytes were scanned for the presence of tin in control, tin(II) and tin(IV) treated cultures. A conservative statistical analysis of the data was employed. Background counts from 3 areas of the spectrum were averaged and then subtracted from the total counts in the combined tin K alpha 1 and 2 peaks. accumulated from the area of interest. To be statistically different from background at the 98% confidence level, net counts had to be greater than $\sqrt{2 \times \text{average background}}$ counts. (Chandler, 1977).

3.5.0. DNA SYNTHESIS INHIBITION ASSAYS.

3.5.1. THE EFFECT OF TIN(II) TREATMENT ON THE ABILITY OF RESTING HPBLs AND MOUSE SPLENOCYTES TO RESPOND TO CONCONAVALIN A.

Human lymphocytes, prepared from freshly collected blood, were suspended in BSS and then treated with various concentrations of tin(II) for 30 minutes at 37°C. After treatment, cells were collected by centrifugation at 400g for 5 minutes, resuspended at 10^6/ml in complete medium containing 10% FCS and conconavalin A (ConA) at 20 ug/ml. Cells were incubated in one ml volumes in 12x75mm vials or in 75cm^2 flasks in an atmosphere of 5% CO_2. Suspensions were pulsed with ^{3}H-TdR 1 uCi/ml for one hour prior to harvesting at 96 hours after exposure of the cells to ConA. Cell number and viability were determined by the trypan blue exclusion test.

Mouse splenocytes, suspended at 2x10^6/ml in RPMI-1640 medium without FCS, were treated with various concentrations of tin(II) and tin(IV) for 30 minutes at 37°C. At the end of the treatments, the cells were collected, washed and resuspended at 2x10^6/ml in medium containing 10% FCS and ConA at 2 ug/ml and incubated for 72 hours at 39°C. Cells were pulse labelled at the end of the incubation period with ^{3}H-TdR (1uCi/ml for 1 hour) and then harvested on GF/c filters.

3.5.2. THE EFFECT OF TIN(II) TREATMENT ON TRANSFORMED AND CON A STIMULATED CELLS, IN VITRO.

Mouse splenocytes were stimulated by ConA for 48 hours and then collected, washed twice and suspended at $2x10^6$/ml in serum-free RPMI-1640 medium. These cells were treated with various concentrations of tin(II) for 30 minutes at 37°C and then transferred to fresh medium containing 10% FCS. The cells were pulse labelled for 20 minutes with ^{3}H-TdR (10 uCi/ml), at various times after the end of the treatment and then harvested onto GF/c filters in a procedure similar to that described by Warren et al.,1980.

Transformed mouse EL-4 cells and human Raji cells were used in a DNA synthesis inhibition assay that is identical to the described by Painter, 1977-1978, for the detection of DNA damaging agents and human carcinogens. Briefly, cells at $2.5x10^5$/ml, were incubated for 30 hours with 0.02 uCi/ml ^{14}C-thymidine (Amersham, 50mCi mmole^{-1}) in complete medium. These prelabelled cells were collected by centrifugation washed once and suspended at 10^6/ml in fresh, nonradioactive medium for 2-3 hours. Cells were then treated with tin(II) at 100, 50 and 1 uM, methylmethanesulfonate (MMS) and hydroxyurea at 1 mM for 30-45 minutes. The cells were again collected by centrifugation, resuspended in fresh medium and distributed to a series of 12x75mm culture tubes. Triplicate tubes of these cells were incubated for 20 minutes in ^{3}H-TdR at various times after the termination of treatment and then harvested onto GF/c filters. In this way the rate of change in DNA synthesis, expressed as the ratio of ^{3}H:^{14}C found in treated cells, was compared to the ratio measured at the same time in controls. The resulting ratios were independent of the cell number in each

culture tube which increased the speed and accuracy of the assay.

3.6.0 FLUORESCENCE ANALYSIS OF DNA UNWINDING (FADU) FOR THE DETECTION OF DNA STRAND BREAK DAMAGE.

The procedure for FADU has been described in detail elsewhere, (Birnboim and Jevak,1981). Briefly, blood was collected in 10 ml vacutainer tubes containing 3.6 mM EDTA as anticoagulant. Blood was pooled and the RBC lysed at $0^{o}C$ The WBC were collected by centrifugation, washed twice in a BSS at $0^{o}C$, resuspended at $2x10^{6}$/ml, treated with various agents and then assayed for DNA strand break damage by FADU.

After the appropriate treatments, the cells were washed twice with solution B (0.25M meso-inositol, 10 mM sodium phosphate buffer and 1 mM magnesium chloride; adjusted to pH 7.2) and resuspended in about 2.8 mls of solution B to give $5-10x10^{6}$ cells/ml. Aliquots (0.2 ml) of this suspension were distributed to 12 disposable glass tubes designated T, P or B, in groups of 4. To each of these were added 0.2 ml of solution C (9M urea, 10 mM sodium hydroxide, 2.5 mM CDTA and 0.1% sodium dodecylsulfate, SDS) and incubated at $0^{o}C$ for 10 minutes. During this time cell lysis and chromatin disruption occurs. To the P and B tubes, 0.1ml of solution D (0.45 volumes of C in 0.2N sodium hydroxide) and 0.1ml of solution E (0.40 volumes of C in 0.2N sodium hydroxide) are very gently added without mixing. During the subsequent incubation at $0^{o}C$ for 30minutes the alkali diffuses into the viscous lysate to give a final pH of about 12.8 (measured at $23^{o}C$). The content of the B tubes are sonicated for 1-2 seconds to ensure rapid denaturation of DNA in the alkaline solution. P and B tubes are incubated at $15^{o}C$ for 60 minutes (or 15 minutes for mouse cells). Denaturation is stopped by chilling to

$0^{o}C$ and the addition of 0.4ml of solution F (1M glucose, 14mM 2-mercaptoethane) with mixing. This lowers the pH to about 11.0. The lysates are sonicated briefly to render them homogenous and diluted with 1.5ml of solution G and their fluorescence read at room temperature in a Farrand Mark I spectrofluorimeter (excitation,520nm; analyzer, 590nm). The T tubes differ from the P tubes in that the neutralizing solution (F) is added before the alkaline solutions D and E so that the DNA is never exposed to a denaturing pH. The extent of the DNA unwinding (ie the amount of duplex DNA remaining) after a given time of exposure of cell extracts to alkali is calculated as P-B/T-B x100 for untreated control cells (Dc) and for treated cells (Dx). The relative amounts of duplex DNA remaining in treated (Dx) and untreated (Dc) cells can be used as a measure of damage produced by the treatment, expressed as -100log(Dx/Dc). The use of 12 tubes permits estimation of percent D in quadruplicate. The B samples are used to estimate the fluorescence due to all cell components other than double stranded DNA; sonication before alkali treatment ensures that the DNA is broken sufficiently to become completely single stranded during exposure to alkali. Up to the point at which solution D is added, all steps are carried out under ordinary room illumination; after this step, manipulations are under subdued light and incubations are in a covered bath. All solutions are kept at $0^{o}C$ except solutions C and G which are kept at room temperature.

3.6.1. THE DIRECT EFFECT OF TIN(II) ON ISOLATED DNA.

Two lots of cells, treated and control, were suspended in 2.8 ml of solution B at $5x10^{6}$/ml, distributed to tubes, as described, and then lysed with solution C. The lysates were treated by standard FADU procedure up until the beginning of the incubation step at $15^{o}C$. At this point, tin(II) was added, in minimum volume

(5 ul), directly to the treatment (P) tubes and M/100 HCl to the P tubes of the controls. The incubation was allowed to proceed for the standard time of one hour and then the lysates assayed for fluorescence as described.

3.6.2. DNA DISASSEMBLY.

Human WBC at 2×10^6/ml, in BSS were treated with 50 uM tin(II), tin(IV) and 25 uM Hg(II). The cells were allowed to incubate at 37°C for 30 minutes before being collected, washed and processed for FADU analysis. Viability of the cells were determined immediately by trypan blue exclusion.

3.6.3 DNA DAMAGE FROM SECONDARY MECHANISMS : THE EFFECT OF THE "OXIDATIVE BURST".

Polymorphonuclear leucocytes (PMNL), a constituent of WBCs, release oxidizing power (in some form) when they ingest particles. This oxidizing burst can be autotoxic and also cytotoxic to the surrounding cells (Tsan et al.,1980). In order to eliminate the possibility that this "burst" may be released as a response of these phagocytic cells to the ingestion of colloidal and particulate tin(II), WBC were incubated in BSS that contained 1 mM 2-deoxyglucose (2-DOG), a nonmetabolizable glucose analogue. This substance is known to suppress the release of the oxidizing burst from stimulated PMNLs. Cells were incubated in the 2-DOG containing BSS for 15 minutes at 37°C and then treated for 30 minutes with tin(II) at 50 uM and several other tin(II) complexes that are known to be colloidal in nature. The cells were harvested and processed for FADU.

3.6.4 THE EEFECT OF TIN(II) AND TIN(IV) ON DAMAGE AND REPAIR OF DNA OF HUMAN WBCs AT 0^{o} AND 37^{o}C.

White blood cells suspended in BSS at $2x10^{6}$/ml, were treated with tin(II) from 5-150 uM, and tin(IV) at 50 uM for 30 minutes at either 37^{o}C or 0^{o}C. in order to establish the initial damage to the cells. A duplicate set of treated cells were washed twice at 0^{o}C in RPMI supplemented with 6% heat inactivated and defibrinated homologous serum and then incubated at 37^{o}C for 20 minutes to study the repair of DNA damage.

Various tin(II) complexes, prepared as previously described, were incubated with the cells at 0^{o} amd 37^{o}C. at a tin(II) concentration of 50 uM. When available, the chelator was used to treat cells as controls. The tin(II) chelates that were assayed were mainly those used in diagnostic nuclear medicine and included; MDP, PyP,MAA, HSA,EDTA, Phytate, F colloid and a variety of phosphates and other substances of nonmedical interest such as the citrate and CDTA.

Human WBC were also treated with a variety of heavy metals and chemicals in an effort to determine the sensitivity of the assay in detecting known DNA damaging agents. The following metals were tested; arsenic, As(III); nickel, Ni(II); cadmium, Cd(II); uranyl, UO_2; vanadyl, VO_2; cobalt, Co(II); lead, Pb(II); zinc, Zn(II); antimony, Sb(III) chromium as chromate, Cr(VI) and chromium as chromic ,Cr(III).

The chemicals were tested at a variety of concentrations and included methylmethanesulfonate (MMS), mitomycin C, cyclophosphamide, bleomycin and

4-nitro-quinoline-N-oxide.

The viability of all treated cells were confirmed by the trypan blue exclusion test 1-1.5 hours after treatment.

3.6.5. THE COMBINED EFFECT OF TIN(II) AND COBALT-60 GAMMA RAYS ON DNA DAMAGE IN HUMAN WBC.

Human WBC were suspended at $2x10^6$/ml in BSS at 37^oC and then divided into 4 equivalent tubes and treated as follows; 1) untreated control cells. 2) cells exposed to 2 Gy of cobalt-60 gamma. 3) cells exposed to tin(II) at 25 uM. 4) cells exposed to both tin and gamma rays.

Tin(II) was added to tubes 3 and 4 and then incubated at 37^oC for 15 minutes and then held on ice while tubes 2 and 4 were being exposed, at 0^oC, to cobalt-60 gamma rays (2.3 minutes at 40 cm). The cells were washed twice in ice cold BSS and then assayed for DNA damage.

3.6.6. THE DETECTION OF DNA DAMAGE IN THE ORGANS OF MICE BY FADU.

Mice/were exposed to cobalt 60 gamma rays at 1,2 and 3 Gy, then immediately sacrificed and their organs (spleen, thymus and bone marrow quickly removed and placed into ice cold BSS. The cells were adjusted to $5\text{-}10x10^6$/ml and then analyzed by FADU using a modified procedure. The length of time at each incubation interval was changed from 10-30-60 minutes to 10-15-15 minutes. The exposure of mice to cobalt irradiation was used to show that damage induced *in vivo* could be detected by the FADU procedure.

To detect *in vivo* damage from tin(II), mice were given intraperitoneal

injections at 7 $mgkg^{-1}$. Control mice were give m/100 HCl. Mice were sacrificed at 1,3,5 and 24 hours after treatment and their spleens excised and processed for FADU by the modified incubation procedure.

3.7.0 ALKALINE SUCROSE GRADIENT ANALYSIS OF DNA DAMAGE IN CHINESE HAMSTER OVARY CELLS.

The procedure is essentially as described by Douglas and and Grant,1980). Briefly, cells ($8x10^5$) were grown in 60mm tissue culture dishes and labelled for 24 hours with ^{14}C-TdR (Amersham 53mCi/mM) at 1uCi/ml or ^{3}H-TdR (Amersham 46Ci/mM) at 1uCi/ml. The cells were then incubated for 4 hours in nonradioactive medium. Cells that were labelled with ^{14}C-TdR were designated as controls and were treated with a 1% solution of the solvent system (M/100 HCl) in minimal essential medium (MEM) without serum. The cells that were labelled with ^{3}H-TdR were similarly treated with 1% solutions of tin(II), tin(IV) and Cr(VI). After the one hour treatment, the cells were washed 3 times in ice cold PBS, removed from the dishes with a rubber policeman and resuspended in 1 ml of ice cold PBS ready for subsequent lysis on alkaline sucrose gradients.

Alkaline sucrose sedimentation was carried out according to the method of Palcic and Skarsgard,1972. Alkaline sucrose gradients, 5-20%, containing 0.3M NaOH, 0.01% SDS, 0.001M EDTA in 4.5 ml, were prepared and a 0.3 ml lysis solution of 0.5M NaOH, 0.2% SDS amd 0.01M EDTA was carefully layered on top. ^{14}C-TdR labelled control cells ($8x10^3$) and ^{3}H-TdR cells ($8x10^3$), in minimum volume (less than 20 ul), was added sequentially to the lysis layer. Lysis was allowed to proceed for 10.5 hours at 20°C before being centrifuged at 15,000rpm for 6 hours in a Beckman SW 50 rotor. Gradients were collected in 0.2 ml fractions and

counted in a liquid scintillation cocktail consisting of 5 ml Aquasol (NEN) and 0.2 ml of 0.5N glacial acetic acid. Calculation of molecular weights was carried out as described by Palcic and Skarsgard,1972.

Cell survival was determined by estimating the relative colony forming ability of the treated cells. About 150 cells were plated in 60 mm tissue culture dishes in MEM with 10% FCS and incubated for 4 hours to allow cells to attach. The cells were treated for 1 hour in serum-free medium at 37°C, washed twice with fresh complete medium and then incubated at 37°C for 7 days. Cells were fixed in ethanol: glacial acetic acid (3:1) and stained with Giemsa and the colonies counted.

3.8.0 TISSUE DISTRIBUTION AND RETENTION PARAMETERS OF TIN(II) IN MICE.

Black C57B6/10 mice, 18-22g were used for these experiments A phantom, made from a plastic centrifuge tube with the approximate dimensions of a mouse, was filled with 20g of water containing 1uCi ^{113}tin(II) and 10ug of carrier tin(II). This phantom was used as a 100% standard and to correct the observed whole body counts in mice for physical decay of the radionuclide. Mice were injected intraperitoneally with 1 uCi of ^{113}tin(II) (10 ug carrier) in 0.2ml of M/100 HCl and then held in metabolic cages to collect the urine and feces. For detection of whole body radioactivity, a mouse was restrained in a vented 50 ml plastic centrifuge tube which was held in fixed geometry over a 5 inch sodium iodide crystal detector. The entire assembly was placed inside of a lead lined cask for sheilding. Counts were accumulated on a TMC 512 multichannel analyzer adjusted so that a 40 channel window was centered at the 393 KeV gamma peak. Counts were then accumulated in the mice at various times after injection. Seven days after the

administration of the ^{113}tin(II), 4 mice were given additional injections of nonradioactive tin(II) and 4 control mice were give equal volumes of M/100 HCl. Whole body counts on this group was accumulated for a further 21 days to determine if the rate of excretion of ^{113}tin(II) was changed by the cold tin(II) injections.

Initially, the radioactivity in the phantom was approximately equal to that in the whole mouse, but with time, the radioactivity remaining in the mouse changes because of physical decay and biological elimination of the radionuclide. If the radioactivity remaining in the mouse is corrected for physical decay then the observed changes will reflect only the biological elimination. This corrected whole body retention curve can then be resolved into its biological components by the standard procedure of curve "stripping" as outlined in Boyd and Dalrymple,1974.

3.8.1 TISSUE DISTRIBUTION OF TIN(II)-113 IN MICE.

^{113}Tin(II), diluted with nonradioactive tin(II) to give a dose of 0.5mgkg^{-1} of body weight, was drawn into syringes and assayed directly for radioactivity in a dose calibrator (Radx Assayer, Radx Corp.), which was sensitive to about 2-3 uCi. The dose was injected into the ventral tail vein of white Swiss-Webster mice (30-33g.) The radioactivity remaining in the syringe after injection was again assayed for radioactivity and the net injected dose was determined for each mouse. Mice were sacrificed in triplicate at 15 minutes, 1, 3, 5, and 24 hours and 3 and 5 days after injection. Various organs were excised and radioactivity was determined either in the dose calibrator or in a well-type NaI crystal detector.

Black mice which were given ^{113}tin(II) by intraperitoneal injection, and were sacrificed in triplicate at 1, 4 and 24 hours and then 2, 5, 10, 15, and 30 days after treatment. Organs were excised and assayed as previously described. The radioactivity in each organ was expressed as a % of the injected dose.

3.9.0 THE EFFECT OF TIN(II) ON THE TISSUE DISTRIBUTION OF ^{99m}Tc-PERTECHNETATE.

White Swiss-Webster mice were treated with tin(II) at 7mgkg^{-1} or M/100 HCl for controls, by intraperitoneal injection, followed in 2-4 days by intraperitoneal injections of 30-50 uCi of carrier-free ^{99m}Tc-pertectnetate. The mice were sacrificed 2 hours later and their organs were excised and assayed for radioactivity. The activity remaining in the whole organ was expressed as a % of the injected dose.

Black C57B6/10 mice (15-17g.) were fed tin(II) at 100ppm in acidified drinking water (adjusted with dilute HCl to give M/100) for a period of 9 months. The drinking supply was changed every 2 days throughout the study period. Mice were given ^{99m}Tc-pertechnetate by intraperitoneal injection 20 hours before sacrifice. Organs of interest were excised and assayed for radioactivity as before.

3.10.0 IMMUNOLOGICAL ASSAYS.

3.10.1 THE ASSAY FOR THE PRIMARY IMMUNE RESPONSE-PLAQUE FORMING CELLS (PFC).

The ability of tin(II) to inhibit the primary immune response *in vivo* to sheep red blood cell (SRBC) antigen was a modification of the Cunningham-Szemberg plaque assay, (Cunningham and Szemberg,1968) Mice were given single doses of tin(II) by intraperitoneal injection (0.5-7.0mgkg^{-1} body weight). This would be about 10-140ug of tin(II) for a 20g mouse. Two days after tin(II) administration, mice were given 0.2ml of a 10% v/v suspension of SRBC in PBS by intraperitoneal injection. The sensitized mice were then sacrificed 5 days later and their spleens quickly excised and made into a cell suspension as previously described. The suspension was adjusted to 10^7/ml in RPMI-1640 and 0.2ml was added to 12x75mm culture tubes followed by 0.15ml of a 25% v/v SRBC suspension, 0.050ml of guinea pig complement (Health and Welfare Canada) and 0.1ml of medium. This mixture was added to Cunningham chambers(25ul/chamber), sealed with petroleum jelly and incubated at 39^{o}C for one hour. The procedure is taken from Dean et al.,1979, Dutton and Mishell,1967 and Mishel and Dutton,1967. The average number of PFCs were determined for 3 replicate slides and expressed as the number of PFC/10^6 splenocytes. The experiments were repeated 3 times.

3.10.2 THE ASSAY FOR ONE-WAY MIXED LYMPHOCYTE REACTION (MLR).

Black C57B6/10 mice were treated by intraperitoneal tin(II) injection at 1, 3, and 5 $mgkg^{-1}$ (a_1,a_3 and a_5) or with M/100 HCl (a_0) as control, for 2 days prior to sacrifice. A crude suspension of spleen cells was prepared and then treated with wheat-germ agglutinin (WGA), (Bourguignon et al.,1979) to obtain an enriched population of T-cells which were then used as responders in one-way mixed cultures. Stimulators consisted equal numbers of balb.c and Swiss-Webster splenocytes which had been treated separately in vitro at 10^7/ml with mitomycin C (40ug/ml) for 30 minutes at 37°C. Responders:Stimulators (1:2)x10^6, were cultured at 39°C in complete medium (supplemented with 50uM 2-mercaptoethanol, 2-ME). One ml suspensions were cultured in 12x75mm plastic tubes and fed daily with 0.1ml of freshly prepared complete medium and 2-ME. Cells were labelled at 72 hours with 1uCi ^{3}H-TdR for 16 hours before being harvested on GF/c filters. In some experiments, tin(II) treated splenocytes were used as stimulators and untreated balb.c splenocytes were used as responders in a one-way MLR. The experiments were repeated 3 times with 2 mice at each dose.

3.10.3 THE EFFECT OF TIN(II) TREATMENT ON DNA AND PROTEIN SYNTHESIS IN THE BONE MARROW AND SPLEEN CELLS OF MICE.

Black C57B6/10 mice were treated by intraperitoneal injections of tin(II) ranging in dose from 0.3-5.0$mgkg^{-1}$ of body weight. Two days later, mice were given intraperitoneal injections of 0.2ml ofa 10% v/v SRBC. Five days after SRBC administration, mice were given ^{3}H-TdR (2uCi/g body weight) by intraperitoneal

injection one hour before sacrifice. Spleens were excised, made into a crude cell suspension and fixed in 1% glutaraldehyde. The suspension was washed with PBS and adjusted to give 10-20x10^6 nucleated cells per ml using gentian violet as stain and hemocytometer counting.. Then 0.2ml of this suspension was dissolved in 0.5ml of Protosol (NEN) and added to a counting cocktail consisting of 0.1ml glacial acetic acid, 0.3ml water and 5ml Scintiverse (Fisher Scientific). Radioactive counts were accumulated for 10 minutes. Bone marrow cells were flushed from both femurs using ice cold medium and processed as above.

In some cases the cells from treated mice were isolated and labelled in vitro for one hour with ^{3}H-TdR at 10uCi/ml. Protein synthesis was also monitored by this in vitro approach using 10uCi ^{3}H-Leu at 10uCi/ml for 2 hours.

11.0 THE STATISTICAL ANALYSIS OF THE DATA.

The student s t-test was used to determine if the differences between means were statistically significant. The formula for the t-test can be found in Freund, 1958.

The statistical analysis of the radioactive counting data was carried out as discussed in the CRC Handbook on Radioactivity, 1969 and Glauert,1975. Correlation coefficients, when used, were calculated according to the method of Armitage, 1971. The coefficient of variation (standard deviation/mean) was used as discussed in Freund,1958. The comparison of multiple means, such as the counts in tin(IV) treated lymphocytes and on colony formation was done using the Duncan s multiple range test as described by Zalik, 1975. The Chi-square was computed according to the method of Lutz,1967.

4.0 RESULTS

4.1 The Uptake of ^{113}Tin(II) by Cells In Vitro

It is important to determine if cells actively accumulate tin(II) and exclude tin(IV) since only tin(II) appears to be active in producing DNA damage. The initial uptake experiments, which involved incubation of various concentrations of ^{113}tin(II) with human WBC for various times, were designed to determine if ^{113}tin(II) was accumulated by the cells, to define and characterize this uptake and to identify the parameters that could modify it.

The results of these initial experiments, shown in Table 6, indicate that uptake at 37°C depends on the concentration of ^{113}tin(II) and on the incubation time. These experiments, which were repeated at 0°C with similar results, indicate that the uptake of ^{113}tin(II) by WBC was not dependent upon the incubation temperature. This suggests that the uptake occurs by a mechanism which did not require the expenditure of cellular energy.

The uptake of tin(II) (Figure 1) by human WBC suspended in BSS, has 2 components; a rapid, saturable phase, which was essentially complete within 5 minutes, and a phase of slow prolonged uptake. The disappearance half-times of ^{113}tin(II) from the external phase of the incubation mixture at 0°C and 37°C varies with the concentration of tin(II) because the contribution of the rapid, saturable component to total uptake is small or non-existent at low concentrations of tin(II). The half-time values of the slow phase of tin(II) uptake were 30 to 40 times longer than those of the rapid phase, Table 7. This is similar to the pattern of uptake for chromate by cultured cells, Ormos and Manyai,1977. The rate constants (K) at 50uM ^{113}tin(II), calculated from the half-time values (K = 0.693/T 1/2) at 0° and 37°C, can be combined to give an estimate for Q_{10} of 1.03 for the uptake process. The Q_{10} is the ratio of the

TABLE 6

THE UPTAKE OF ^{113}TIN(II) BY HUMAN WHITE BLOOD CELLS IN BALANCED SALT SOLUTION AT 0°C AND 37°C.

^{113}Tin(II) Concentration (uM)	^{113}Tin(II) Bound (fM) per Cell			
	0°C		37°C	
	5 min.	30 min.	5 min.	30 min.
50	8.00±0.37	8.42±0.22	7.27±0.42	7.66±0.55
25	n.d.	n.d.	2.42 0.18	2.72 0.75
10	0.50 0.08	0.48 0.08	0.36 0.04	0.44 0.10
5	0.15 0.02	0.15 0.07	0.11 0.01	0.12 0.02
1	0.02 0.00	0.02 0.01	0.03 0.01	0.03 0.01

TABLE 6 UPTAKE OF ^{113}TIN(II) BY WBC. The uptake of ^{113}tin(II) was measured at various incubation times by the rapid centrifuge technique of Strauss et al, 1977. Uptake was essentially complete after 5 minutes of incubation but a slow phase of prolonged accumulation was also evident at concentrations 5uM. The difference in uptakes at 0°C and 37°C was not significant at p=0.02 suggesting that the accumulation of ^{113}tin(II) by cells was a passive process.

Fig. 1

Uptake of ^{113}Tin (II) by White Blood Cells in Balanced Salt Solution at 37°C

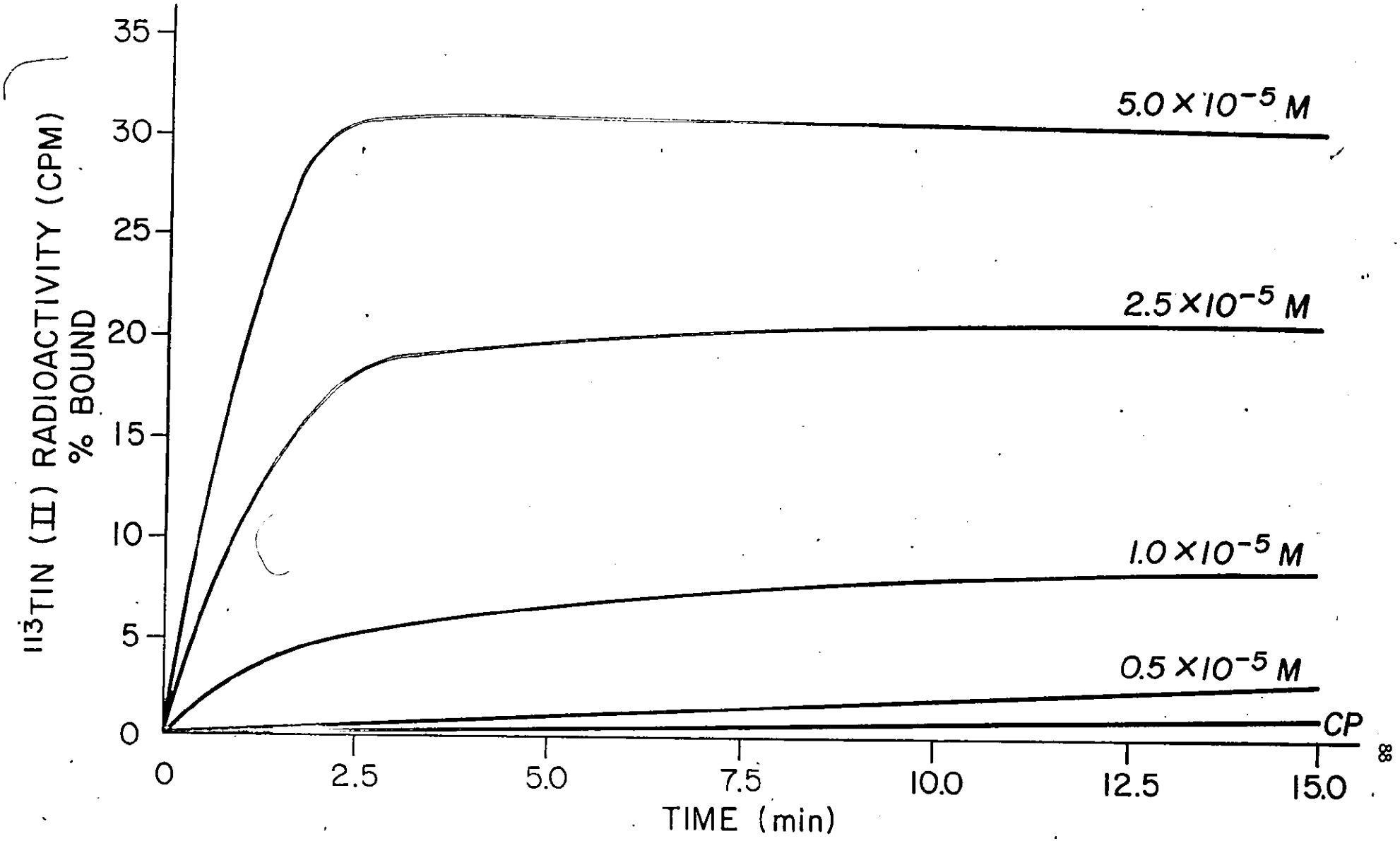


Figure I. The uptake of ^{113}tin(II) by white blood cells in balanced salt solution at 37^{o}C. A slow prolonged uptake of radioactivity is apparent with extended incubation times. The addition of 100 uM nonradioactive tin(II) to the suspension, 5 minutes after the start of incubation, didnot displace any radioactivity from the cells. Instead, uptake again increased until an apparent saturation was attained (data not shown). These experiments were also carried out at 0^{o}C with similar results.

TABLE 7

THE RATES OF UPTAKE (K) INTO HUMAN WHITE BLOOD CELLS AND THE CLEARANCE HALF-TIMES ($T_{\frac{1}{2}}$) OF ^{113}TIN(II) FROM BALANCED SALT SOLUTION AT 0°C AND 37°C.

Tin(II) uM	Incubation Time 5Min. K (Min.-1)	5Min. $T_{\frac{1}{2}}$ (Min.)	30Min. K (Min.-1)	30Min. $T_{\frac{1}{2}}$ (Min.)
0°C				
50	0.076	9.1	0.002	347
-n.d.-				
10	0.020	34.1	0.004	173
5	0.012	57.8	0.002	347
1	0.010	69.3	0.002	347
37°C				
50	0.070	9.9	0.002	347
25	0.044	15.8	0.002	347
10	0.021	33.5	0.002	347
		72.1	0.001	693
		66.3	0.001	693

TABLE 7. THE HALF-TIME VALUES FOR THE DISSAPPEARANCE OF ^{113}TIN(II) FROM THE INCUBATION MEDIUM AT 0°C and 37°C. The uptake of radioactivity into cells was monitored at various times by the centrifuge method of Strauss et al. 1977. The rates of uptake and the clearance half-times of tin(II)-113 into cells were calculated according to the methods outlined by Lehninger 1970 and Ormos 1977. These values were found to depend on the concentration of tin(II)-113 in the incubation medium, which is characteristic of a first order reaction process.

FIGURE 2

Saturable Component of ^{113} Tin (II) Uptake in Human White Blood Cells in Balanced Salt Solution at 37°C

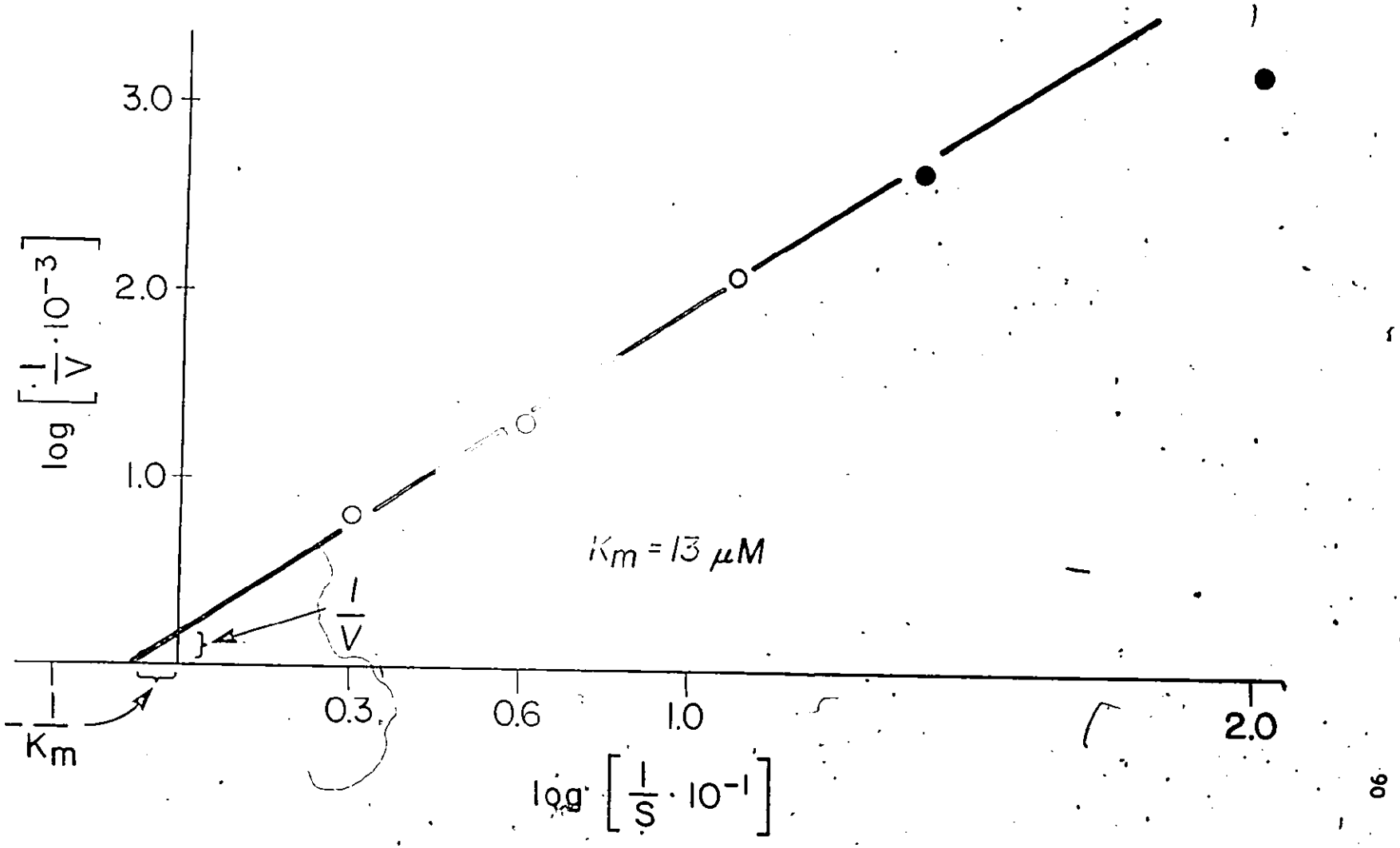


Figure 2. Saturable tin(II) uptake in human white blood cells at 37°C. The uptake of radioactive tin(II) was essentially complete within 5 minutes at each of the tin(II) concentrations studied. The Km and Vmax for this component of total uptake was calculated from the reciprocal plot to be I3 uM and 3.56fM/cell respectively. A Km of 22 uM was calculated from the direct plot of this data (Wold,I97I) and combined with the results from the reciprocal plot to give an estimate of I7.5 uM. The Km indicates the concentration of tin(II) required to occuppy half of the total available binding sites. Interpolation from Table 6 indicates that I7.5 uM tin(II) in the incubation mixture would give an uptake of about 2.56 fM/cell. The total number of binding sites available to tin(II) per cell then is about 3.1×10^9, of which half would be associated with the nucleus.

velocity constant of a process or reaction at a given temperature and concentration to the velocity constant at a temperature 10°C higher (see Heilbrunn, 1958 for a discussion). It is widely believed, although there are many exceptions, that the Q_{10} for a physically controlled process is < 2 or > 3 (van't Hoffs rule). The fact that cells accumulate tin(II) equally well at 0°C and 37°C is a more reliable indication that the mechanism of uptake is by a physically controlled process, such as passive diffusion.

Data from Figure 1, referring to the rapid (5 minute), saturable phase, can be used, in a reciprocal plot (Figure 2), to estimate the maximum velocity (V_{max}) of ^{113}tin(II) uptake and the tin(II) concentration (K_m) at which the uptake is half maximum, Wold, 1971. An analysis of the reciprocal plot of velocity of uptake (log 1/V) against tin(II) concentration (log 1/S) (Figure 2), give estimates for K_m of 13 μM and V_{max} of 3.56 fM/cell/minute. The data was also analyzed by a direct plot (Wold, 1971) to give an estimate for K_m of 22 μM (data not shown). Mouse splenocytes, suspended in BSS or RPMI-1640 without FCS at 0°C or 37°C displayed a similar uptake pattern.

4.1.2 The Effect of Chelating Agents on the Uptake of ^{113}Tin(II) by Human WBC In Vitro

The uptake of the ^{113}tin(II) chelates of ethylenediaminetetraacetic acid (EDTA), trans-1,2, diaminocyclohexane -N, N, N', N'-tetraactic acid (CDTA), pyrophosphate (PyP), methylene diphosphonate (MDP), human serum albumin (HSA) phytate and fluoride (F^-) colloid by human WBC suspended in BSS at 0°C and 37°C is shown in Table 8. These chelating agents have a range of affinities for tin(II) (Table 2). EDTA, which does not penetrate the cell membrane (Rahman, 1979) binds strongly to tin(II), (log binding constant, K = 18) and prevents its uptake by the cell at both temperatures. The presence of a protein (HSA) in the

TABLE 8

THE EFFECT OF INCUBATION TEMPERATURE ON THE UPTAKE OF TIN(II) CHELATES BY HUMAN WHITE BLOOD CELLS SUSPENDED IN BALANCED SALT SOLUTION..

Treatment	Uptake after 30 minute incubation (fM bound/cell)	
	0°C	37°C.
Tin(II) Chloride	8.42	7.66
Tin(II) Fluoride colloid	0.70	3.42
Tin(II) EDTA	0.03	0.02
Tin(II) PyP	0.70	2.37
Tin(II) MDP	0.03	0.18
Tin(II) Phytate	15.50	16.75
Tin(II) CDTA	2.37	1.36

TABLE 8. THE EFFECT OF TEMPERATURE ON THE UPTAKE OF TIN(II) CHELATES BY HUMAN WHITE BLOOD CELLS IN A BALANCED SALT SOLUTION. The uptakes of tin(II) chloride, phytate and CDTA were independent of the incubation temperature. Uptakes for these compounds however, were found to be consistently greater at 0°C, which could be due to the inactivity of cell pumps at this temperature. The accumulation of tin(II) fluoride colloid, Pyp and MDP was by and energy dependent process, being typioally much greater at 37°C. Tin(II) EDTA didnot penetrate the cell membrane. Uptakes were determined by the rapid centrifuge technique of Strauss et al. 1977.

incubation mixture prevents the uptake of tin(II) at 0°C and severely hinders uptake at 37°C. ^{113}Tin(II) forms a complex with HSA that can be precipitated from solution by the addition of 5% trichloroacetie acid (TCA) (data not shown). This means that the tin(II) HSA complex could slowly gain entry to the cells by the process of pinocytosis, Pratten et al, 1977. The internalized metal-protein complex would be metabolized in the lysosomes and tin(II) would be gradually released into the intracellular space where it could damage DNA. A latent period would occur between initial exposure and observed damage because uptake is slow by pinoytosis and digestion of the HSA carrier is necessary to release the tin(II). Cells, in medium containing 6% FCS, must be exposed to tin(II) for about 12 hours at 37°C before DNA damage can be detected (data not shown), indicating that reactive tin(II) becomes available with increasing incubation times.

Tin(II) strongly binds to PyP and the cellular uptake of this complex is energy dependent, occurring to a greater extent at 37°C than at 0°C. This is also the case with tin(II) MDP and tin(II) fluoride colloid, and is the pattern that would be expected if uptake were by phagocytosis or active transport. At neutral pH, tin(II) PyP and tin(II) MDP are solutions whereas tin(II) fluoride is a colloid with particles ranging in size up to 12 nm (Amersham, private communication).

The cellular uptake of tin(II) is enhanced by the presence of phytate (inositol hexaphosphate) in the incubation medium. Binding of tin(II) to phytate probably occurs through the phosphate moeity and would be relatively weak (log K = 3 phosphate). Several tin(II) atoms could theoretically bind to each phytate molecule. This would reduce the net charge on the complex and would facilitate penetration of the cell membrane by the complex. The uptake of Tin(II) phytate by cells is slightly greater at 0°C than at 37°C because cell pumps would be

active at the higher temperature and would remove intracellular tin(II) phytate that was not firmly bound to macromolecules.

Tin(II) binds very strongly to CDTA (log K = 18) and this complex, like Pt-CDTA is rapidly taken up by the cells. Unlike highly charged EDTA, CDTA contains a saturated ring structure and a low net charge and these properties would allow the tin(II) complex to penetrate the cell membrane more readily than tin(II)-EDTA.

4.1.3.0 The Subcellular Distribution of Tin(II) and Tin(IV)

Once it was established that tin(II) accumulated in cells, the next step was to identify the intracellular binding sites which could be the sensitive targets. These preliminary subcellular distribution studies were designed to determine if tin(II) could act directly on DNA. The association of tin(II) with cell nuclei would strengthen this contention. A secondary effect on DNA (DNA disassembly) would be more likely if tin(II) was associated with only the cell membranes or cytoplasm.

4.1.3.1 Subcellular Fractionation of ^{113}Tin(II)

Mouse splenocytes bound 25% of the radioactivity when they were incubated for 15 minutes at 37^{o}C in BSS containing 50 μM ^{113}tin(II). The nuclear fraction, consisting of intact nuclei isolated from cells by gentle lysis, contained 57% of the cell bound radioactivity or 15% of the total radioactivity in the incubation mixture. The crude membrane and cytoplasmic fraction contained 43% of the cell associated radioactivity. The total ^{113}tin(II) activity bound by cells in the presence of 10% FCS under similiar conditions was reduced from 25% to 8%. This preliminary result suggests that ^{113}tin(II) is rapidly taken up by the cell and is more or less evenly partitioned between cytoplasm and nucleus.

There is no isotope of ^{113}tin(IV) and therefore X-ray fluorescence was used to determine the subcellular distribution of tin(IV) and to confirm the distribution of tin(II).

4.1.3.2 X-ray Microbeam Analysis of Human Peripheral White Blood Cells.

Microbeam analysis,which uses standard EM procedures for specimen preparation, detects only that tin(II) which is firmly bound to the macromolecules and organelles of the cell. The centrifuge technique which was used to study the binding of ^{113}in(II), gives a measure of the radioactivity that is bound to cell constituents with a range of affinities. Therefore,the two uptakes are not comparable.

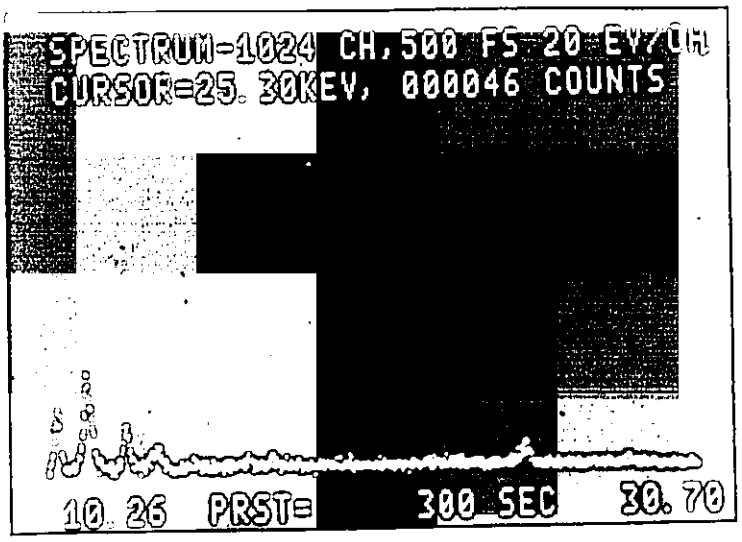
Microbeam analysis of WBC treated in BSS at 37^{o}C for 30 minutes with tin(II) or tin(IV) at 100uM, clearly allows the characteristic X-rays of tin to be identified by the combined alpha peaks (Ka-1 and Ka-2 at 25.3 and 25.0 KeV respectively) and Kb (28.5 Kev) in photographs 1-4,Weast 1976.

Photograph 1A shows a colloidal, electron dense particle being taken into the cell from an incubation mixture containing tin(II). Microbeam analysis of a restricted area (0.2um dia.) around the particle clearly identifies it as tin, photograph 1A. Lymphocytes and polymorphonuclear leucocytes (PMNL) take up tin(II) equally well (photographs 2, 3, and 4). The semi-quantitative nature of this uptake is described in table 9. The radioactivity from the characteristic x-rays in the tin(II) preparations, rows 1(a), 1(b) and 2 (table 9) were statistically different from background at the 98% level of confidence.Relative counts detected in the cytoplasm and in the nucleus were about equal, confirming the preliminary observations obtained from the subcellular distribution studies of ^{113}tin(II) using the centrifuge technique.

RBC did not accumulate tin(II), confirming isotope studies (data not shown)

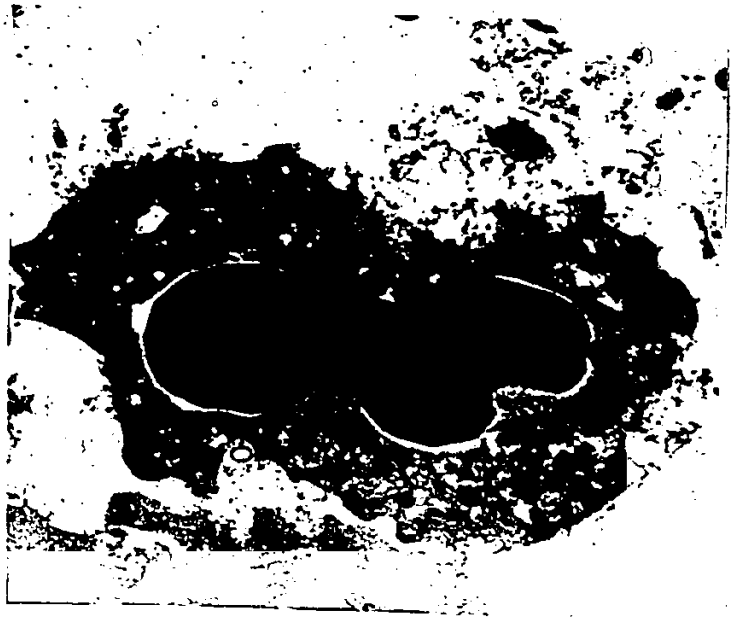
PHOTOMICROGRAPH 1. A mononuclear leucocyte ingesting a particle of tin(II), x12,000. A microprobe, with a beam diameter of 0.2µm, was placed over the area indicated by the arrow. The characteristic x-rays of the tin was collected on a multichannel analyzer for 300 seconds and then displayed as a spectrum of accumulated counts (photo 1b). Many smaller particles of tin(II) can be seen inside and at the periphery of the cell. The size of the tin(II) particles, as a first approximation, ranged up to about 40nm.

PHOTOMICROGRAPH 1A

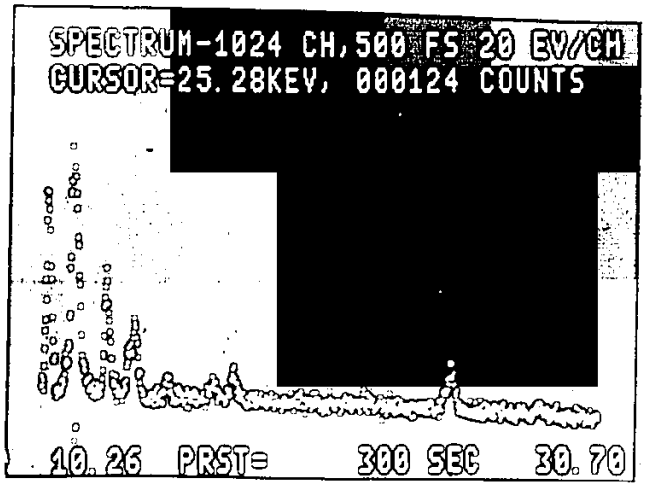


PHOTOMICROGRAPH 1B

PHOTOMICROGRAPH 2. A human polymorphonuclear leucocyte (PMNL) ingesting tin(II) particles x 16,000. The microprobe, 0.5µm, was placed over the nuclear area in the long lobe. The cells were only lightly fixed in glutaraldehyde which causes the nuclear membrane to contract after the dehydration step just prior to embedding in Spurr.



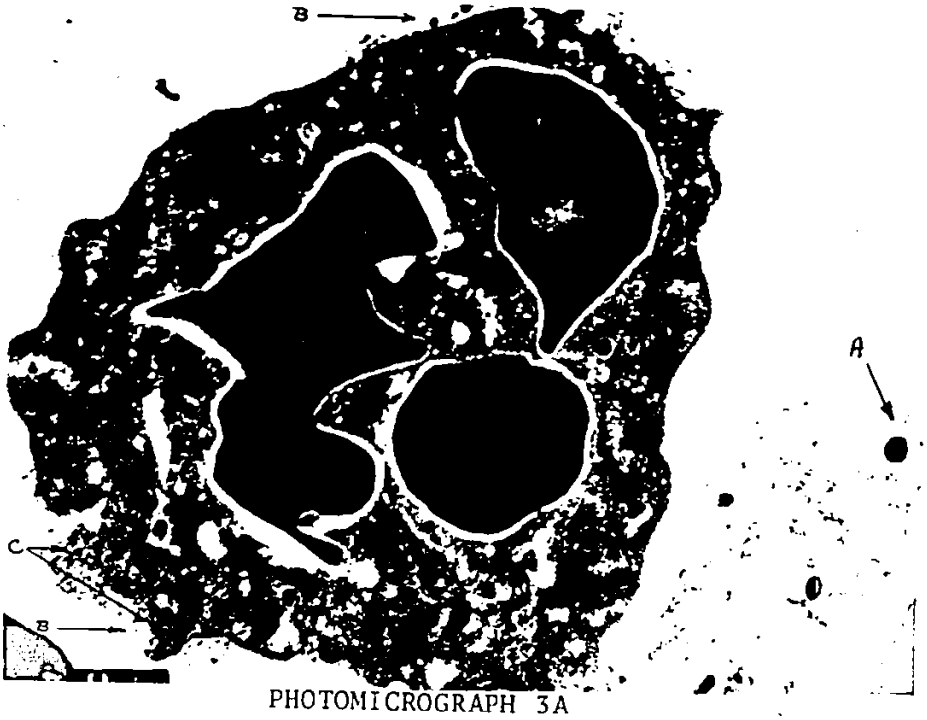
PHOTOMICROGRAPH 2A



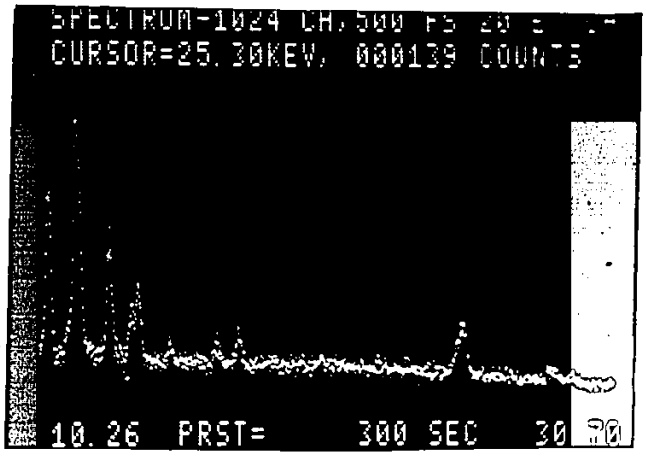
PHOTOMICROGRAPH 2B

PHOTOMICROGRAPH 3A. Polymorphonuclear leucocyte x16,000. Arrow at A indicates a large, 0.25 um diameter particle of tin. Arrow at B indicates a 40 nm diameter particle of tin at the cell periphery and inside the cytoplasm at C. Photograph at 3B shows radioactive counts accumulated in a 0.5 um diameter area of the circular lobe of the nucleus relative to an area outside of the cell.

PHOTOMICROGRAPH 4. PMNL x 6,000. Arrow shows a large accumulation of tin inside of the cell. Cells were not treated with osmium and were stained only lightly with uranyl and lead solutions. The control cells (no tin) lacked the electron dense areas or granules found in those cells that were treated with tin(II).



PHOTOMICROGRAPH 3A



PHOTOMICROGRAPH 3B

PHOTOMICROGRAPH 4

TABLE 9

DETECTION OF TIN IN HUMAN WHITE BLOOD CELLS

BY MICROBEAM ANALYSIS.

Area of Interest	Detection Limit ($\sqrt{2}$ BKG)	Net Counts (300sec.) Tin(II)	Tin(IV)
1.Nucleus			
a.Lymphocyte	15	70	18
b.PMNL	15	81	
2.Cytoplasm (lymphocyte)	9	36	
3.RBC	11	5	

TABLE 9.DETECTION OF TIN IN HUMAN WBC BY MICROBEAM ANALYSIS. A beam current of 3uAmps, 0.5um in diameter (0.80um^2) was used to irradiate selected areas of the cells. Radioactivity of the combined K-alpha 1 and 2 tin characteristic x-ray peaks were accumulated in a multichannel analyzer for 300 seconds. Tin characteristic x-rays were readily detected in the cytoplasm and nuclei of lymphocytes and PMNL after tin(II) but none was detected in cells after treatment with tin(IV). To be statistically significant at the 98% level net counts had to be $\sqrt{2}$ times the detection limit.

and animal studies, Hiles, 1974 and Hamilton et al, 1972.

Tin could not be detected in cells treated with tin(IV) even though it was detected in cellular debris and as fiberous particles outside of the cells.

4.2 The Cellular Uptake of Tin (II) and the Inhibition of DNA Synthesis In Vitro.

In this series of preliminary studies, the uptake of tin(II) by the cell is related to the suppression of DNA synthesis. Study of this effect *in vitro* is useful because parameters can be easily manipulated and related to a clearly observable phenomenon, a process which is more complicated when studied *in vivo*.

Previous studies have shown that cells rapidly accumulate tin(II), probably by a passive mechanism. The tin(II) that is taken up by the cell is evenly partitioned between cytoplasm and nucleus. This means that tin(II) could exert its effect on the cell membrane or at numerous intracellular sites. In contrast, tin(IV) is excluded from the cell as indicated by microbeam analysis. The effect of tin(II) and tin(IV) treatments on DNA synthesis was also used to identify the intracellular compartments containing the targets that are sensitive to the action of tin(II). As previously discussed, there is an empirical relationship between the ability of an agent to produce DNA damage in cells and its ability to produce a prolonged suppression of DNA synthesis, Painter 1977, Painter 1978 and Warren et al 1980. Agents that suppress DNA synthesis by a metabolic action exert their effect only for as long as they are present in the incubation medium. The effects observed after treatment by the agents is usually the result of reaction with sites on the membrane or in the cytoplasm. Therefore, these studies not only document a potential threat to

health but also help to identify the site of the primary lesion responsible for the observed biological effect. These studies were carried out by examining the effect of tin(II) on DNA synthesis in lymphocytes stimulated by Con A, in transformed mouse EL-4 and human lymphoid cells and finally in lymphoid cells treated in vivo, where the effects, if prolonged, could threaten the health and well-being of the organism.

4.2.1. The Effect of Tin (II) and Tin (IV) on DNA Synthesis in Lymphoid Cells Stimulated by Con A.

The pretreatment of HPBL with tin(II), suspended in BSS for 30 minutes, produced a graded inhibition of blastogenesis with increasing dose, Table 10 . Interpolation from Table 10 indicates that treatment of HPBL with tin(II) at 20uM for 30 minutes in serum-free medium would inhibit blastogenesis by 50%, as measured by the incorporation of ^{3}H-Thymidine into cells, at 96 hours after the addition of Con A and FCS.

The lymphoid cells from mouse spleens were used to confirm this effect of tin(II) on DNA synthesis and to document the effect of tin(IV) treatments. Mouse splenocytes, suspended in BSS or serum-free RPMI-1640 containing 50uM tin(II)-113, take up an average of 7.86fM/cell after a 30 minute incubation at cell densities ranging from 0.25-3.0x10^{6} per ml (data not shown),similar to human WBC, table 6. A brief one hour pretreatment of mouse splenocytes with tin(II) or tin(IV) in serum-free RPMI-1640 medium was sufficient to produce a statistically significant inhibition of DNA synthesis for tin(II) but not for tin(IV) after 72 hours of incubation in fresh medium supplemented with FCS and Con A,figure 3. Interpolation from figure 3, indicates that a tin (II) concentration of 30uM would inhibit the rate of DNA synthesis by 50%. Pretreatment of mouse splenocytes with tin(II) also inhibited protein and RNA synthesis but to a lesser extent than DNA synthesis (Data not shown).

These experiments indicate that tin(II) suppresses DNA synthesis in Con A stimulated mouse splenocytes and human PBL to the same extent. An empirical

Fig. 3

Effects of Tin (II) and Tin (IV) Pretreatments on DNA Synthesis in Conconavalin a Stimulated Mouse Splenocytes

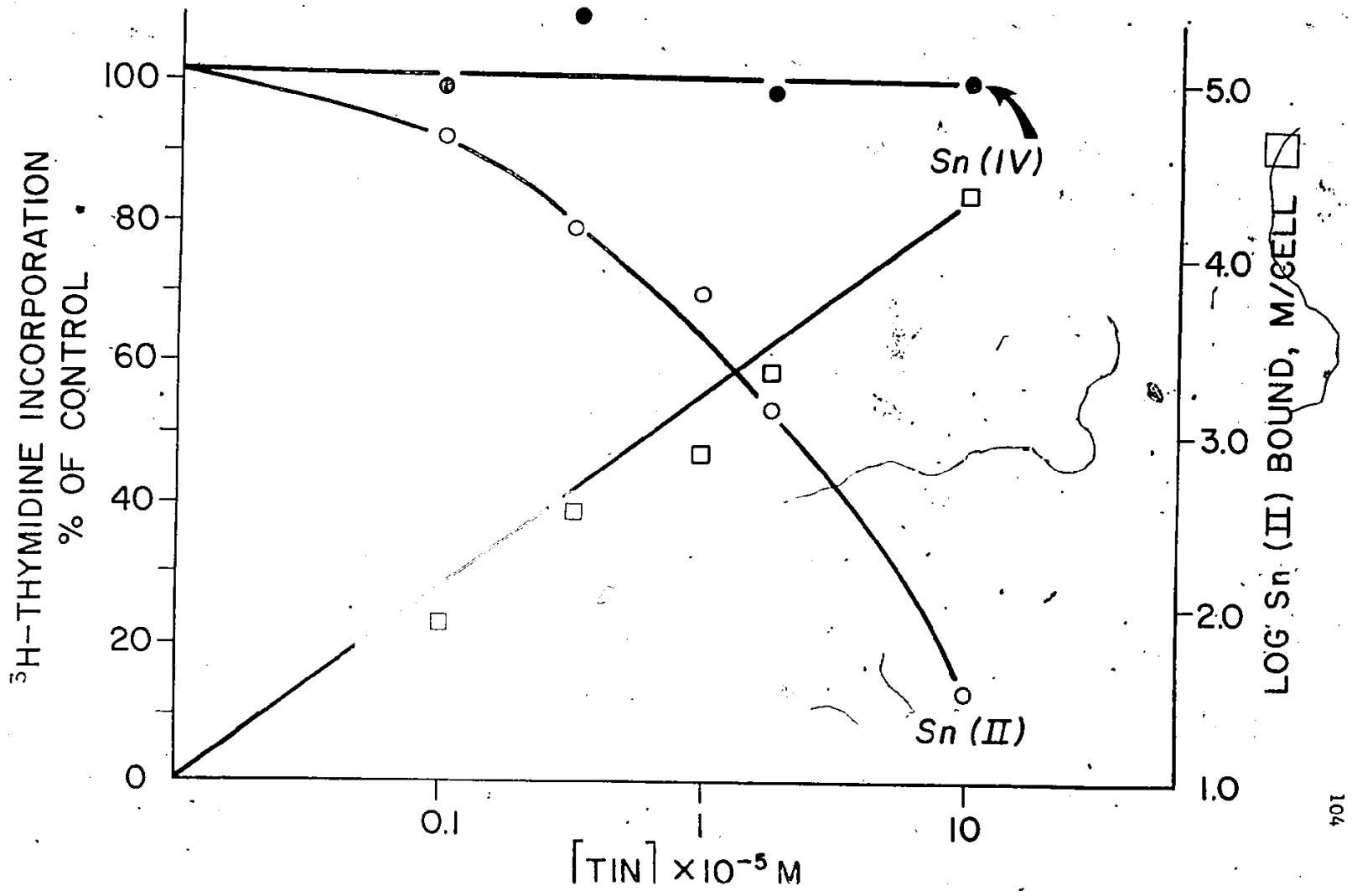


Figure 3. The effects of tin(II) (o) and tin(IV) (●) on DNA synthesis in ConA stimulated mouse spleen cells. Mouse cells were treated for one hour in serum-free medium at 39°C. Some cells were incubated with ^{II3}tin(II) and assayed for cell bound radioactivity (□). Cells from the nonradioactive treatments were resuspended in fresh medium with ConA and fetal calf serum and the incubated at 39°C. The rate of DNA synthesis was determined at 72 hours by measuring the incorporation of ^{3}H-thymidine into the acid insoluble fraction of the cells.

relationship can be established between the degree of DNA synthesis inhibition and the amount of tin(II) taken up by the cell. The fact that a brief pretreatment of cells by tin(II) is sufficient to irreversibly inhibit DNA synthesis, suggests that the reaction of tin(II) with some critical target in the cell produces long-lived lesions which act as barriers to either the initiation or elongation of the DNA replication sites.

4.2.2. The Effect of Tin (II) on DNA Synthesis in Transformed Cells.

The experiments showing that brief treatments of cells with tin(II) bring about a prolonged suppression of DNA synthesis suggests that DNA could be the critical target. Damage to proteins or RNA would produce only a transient suppression of DNA synthesis because an intact DNA template would be able to replace these damaged or inactivated molecules. The DNA synthesis inhibition assay using HeLa cells was described by Painter in 1977 as a rapid screening method for the detection of DNA damaging agents and human carcinogens. Briefly, agents that damage DNA produce a rapid and prolonged suppression of DNA synthesis while agents that are metabolic inhibitors produce only a transient perturbation under the conditions that are employed in the assay, Painter 1977, 1978 and Warren et al, 1980. The information gained from this inhibition assay supplements the observation that tin(II) produces a prolonged suppression of blastogenesis in cells stimulated by Con A and further supports the contention that tin(II) induces long lived lesions directly into the DNA of treated cells.

TABLE 10

THE EFFECT OF TIN(II) TREATMENT ON DNA SYNTHESIS IN HUMAN LYMPHOCYTES AFTER EXPOSURE TO CON A FOR 96 HOURS.

Treatment	Incorporation of ^{3}H-Thymidine	
Tin(II),uM	CPM/10^{6}Cells	%Control
Control	631	
ConA	60,000	100.0
ConA plus		
100	7,018	11.7
50	14,784	24.6
20	39,802	66.4
10	52,030	86.8
5	57,600	96.0

TABLE 10 THE EFFECT OF TIN(II) ON BLASTOGENESIS IN HUMAN LYMPHOCYTES. After a 1 hour tin(II) treatment in serum-free medium, cells were incubated in the presence of serum and ConA for 95 hours. At the end of this incubation period, the rate of DNA synthesis was determined by assessing the incorporation of ^{3}H-thymidine into the acid insoluble fraction of cells. The lower confidence level for the ConA stimulated controls was 53,500 cpm/10^6 cells, calaculated at p=0.005, Freund 1958. A graded inhibition of blastogenesis occurred with increasing concentrations of tin(II). The nature of the tin(II) induced lesions, which act as barriers to DNA synthesis, is not known but several hypotheses can be extended. The lesions may be repaired with difficulty by the existing enzymes or repair could be inhibited by the presence of tin(II). Alternately, the firmly bound intracellular tin(II) could remain reactive in-situ and continuously generate lesions in DNA and although capable of being quickly repaired their frequency would disrupt the conformation of the DNA and hence the replication process.

4.2.3 The Effect of Tin(II) on DNA Synthesis in Human Raji Cells and Mouse EL-4 Cells.

The assay described by Painter, 1977 was modified to use human Raji cells and mouse EL-4 cells, two transformed lymphoid lines. The results shown in figures 4 and 5 , indicate that brief pretreatment of these cells with tin(II) produced a prolonged and rapid inhibition of DNA synthesis. The Raji cells were treated for 30 minutes in the presence of 6% FCS while the mouse EL-4 cells were treated for 60 minutes in serum-free RPMI-1640 medium, which accounts for the difference in tin(II) concentrations that were required to inhibit DNA synthesis by 50%. In Raji cells, 100uM tin(II) was required to inhibit DNA synthesis by 50%. The rate of DNA synthesis was suppressed for a prolonged period in Raji cells treated with MMS after the cells were reincubated in fresh medium but the rate quickly returned to control levels in those cells treated with hydroxyurea, figure 6. This same type of response was found by Painter for these agents using HeLa cells, (Painter, 1977).

Mouse EL-4 cells were treated at a much lower tin(II) concentrations and for 60 minutes in RPMI-1640 without FCS. Binding of ^{113}tin(II) to mouse EL-4 cells under these incubation conditions is shown in table 11 and dose-effect curves, at 3 and 24 hours after treatment, are shown in figure 7. Interpolation from figure 7 indicates that a one hour treatment of EL-4 cells with 1.3uM tin(II) would be required to inhibit the rate of synthesis by 50% after 3 hours of incubation whereas 0.6 uM would inhibit by 50% after 24 hours. These 50% inhibition levels correspond to an initial binding of 0.09 and 0.02 fM/cells and an average residual binding of 0.06 and 0.017 fM/cell at 24 hours after treatment. The rate of loss of tin (II) from cells was about 0.5% of the bound radioactivity/hour.

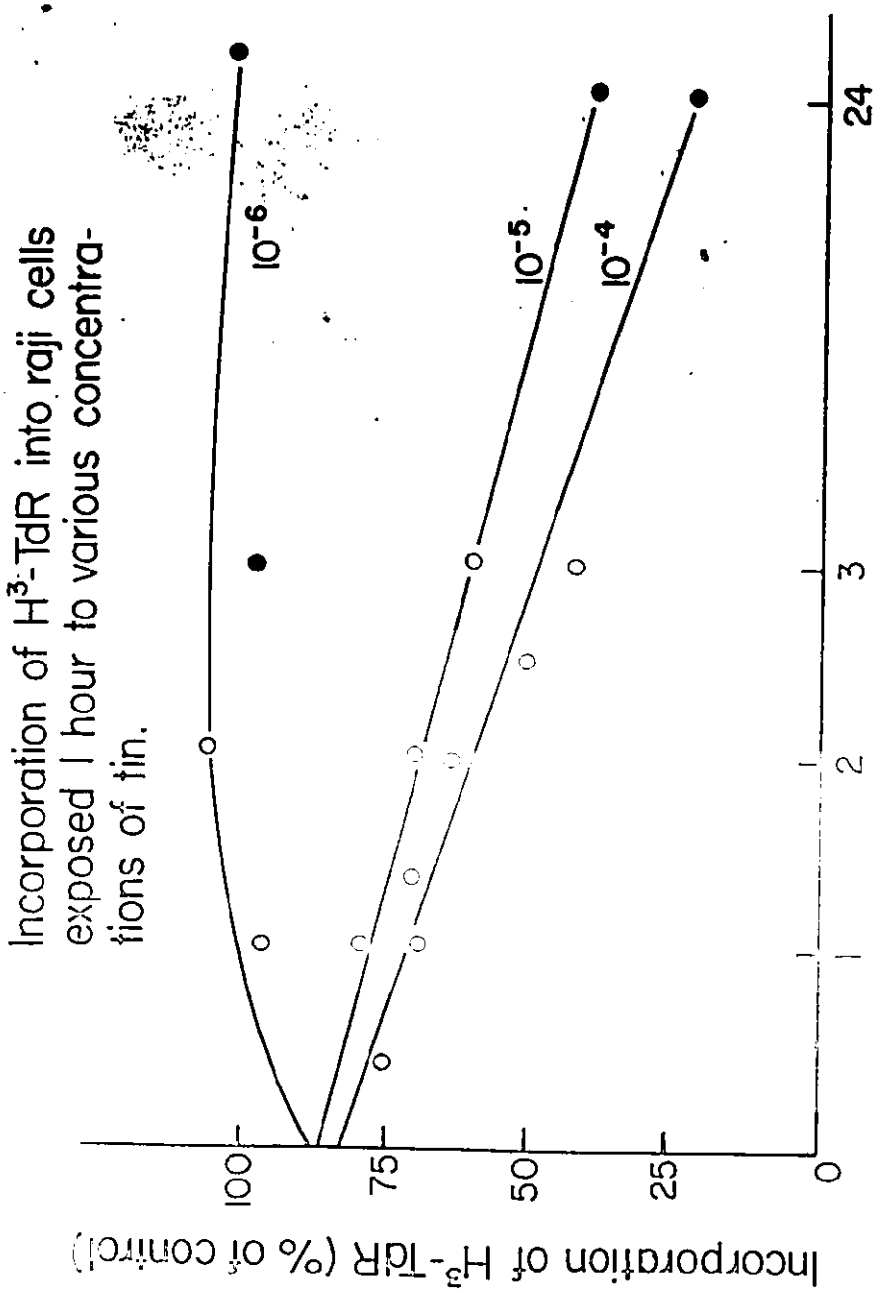
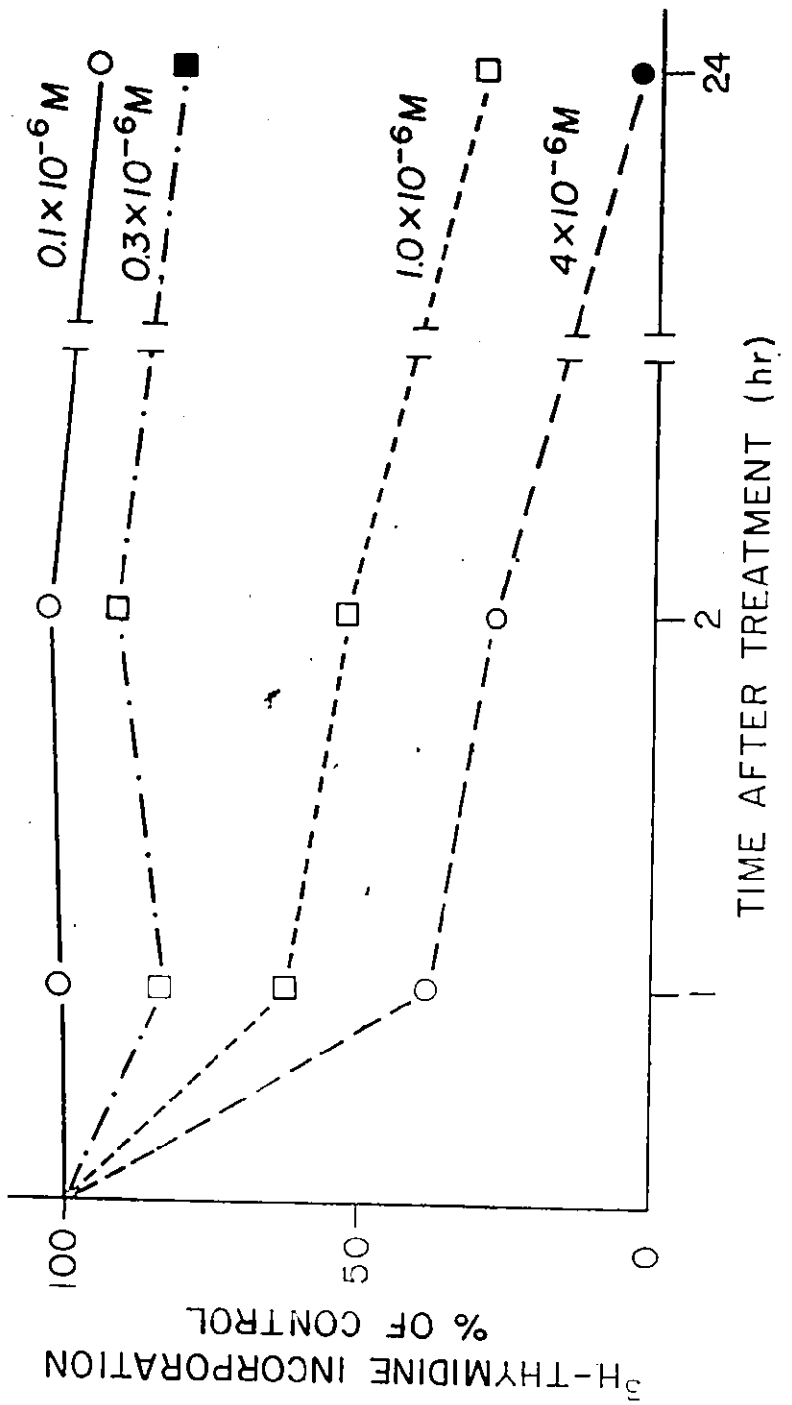

Incorporation of H³-TdR into raji cells exposed 1 hour to various concentrations of tin.
10⁻⁶
10⁻⁵
10⁻⁴
Incorporation of H³-TdR (% of control)
100
75
50
25
0
1
2
3
24
Time (hours)

FIGURE 4

Figure 4. Incorporation of ^{3}H-thymidine into Raji cells exposed one hour to various concentrations of tin(II). Cells were exposed to tin(II) in the presence of FCS, washed twice and the rate of DNA synthesis monitored at various times by ^{3}H-thymidine incorporation. Agents that produce a rapid and prolonged suppression of DNA synthesis have been correlated with DNA damage(Painter 1977 and Warren 1980). The inhibition at 10^{-6}M is transient suggesting that tin(II) may not have been able to penetrate the nucleus in sufficient concentration to produce extensive DNA damage. Tin(II) however, could exert a reversible effect on DNA synthesis by degrading RNA, like some other divalent metals (Butzow,1965).

Fig. 5

Rate of DNA Synthesis in Mouse EL-4 Cells
Pretreated With Tin (II)



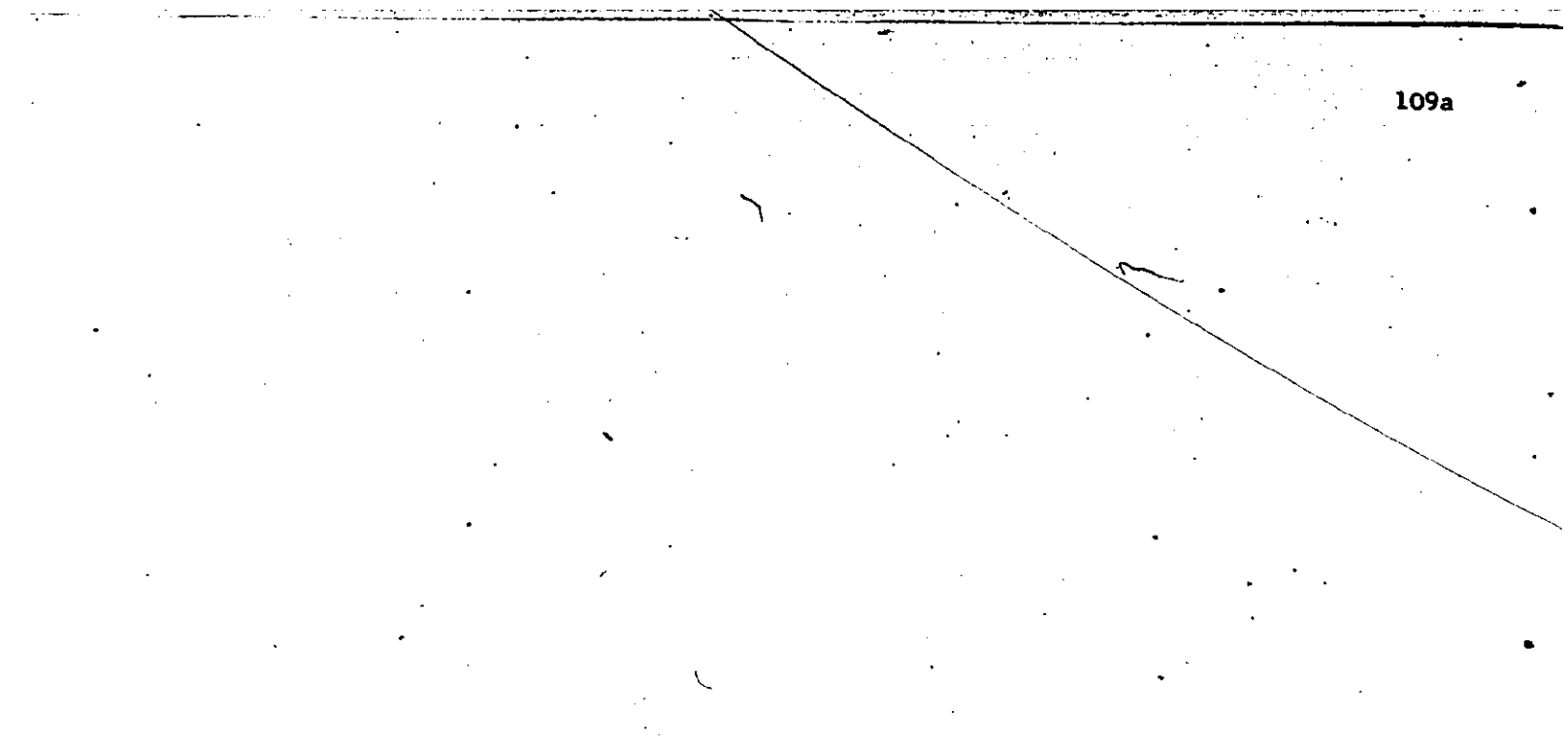


Figure 5. The rate of DNA synthesis in Tin(II) treated mouse EL-4 cells. Mouse EL-4 cells, a chemically transformed lymphoid cell line, were treated for 60 minutes in serum-free medium with various concentrations of tin(II). Irreversible inhibition of DNA synthesis occurred at treatments as low as 0.3 uM ( ) which represents 0.5 fM bound per cell.

FIGURE 6

Incorporation of H^3-TdR in raji cells exposed 1 hour to HU and MMS.

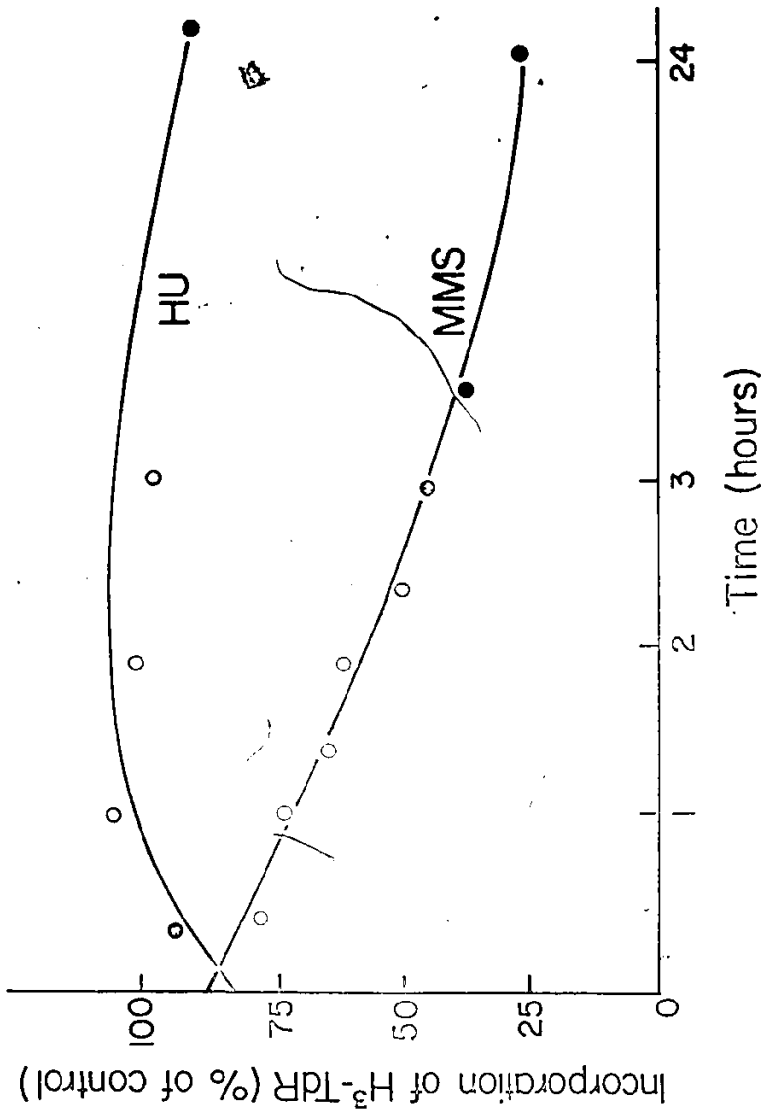


Figure 6. Incorporation of ^{3}H-thymidine into Raji cells exposed for one hour to hydroxyurea and methylmethane sulfonate. Cells were treated and assayed essentially as described by Painter 1977. The response of the Raji cells were similar to that of the HeLa cells used by Painter, suggesting that the two assays give similar results for these substances.

TABLE 11

BINDING OF 113TIN(II) TO MOUSE EL-4 CELLS AT VARIOUS TIMES AFTER TREATMENT.

Treatment Tin(II), uM	Initial Binding	Residual Binding, fM Tin(II) per Cell		
		Time (Hr.) After Treatment		
		1	2.5	24
4.0	0.62	0.62	0.63	0.55
1.0	0.15	0.15	0.15	0.14
0.3	0.05	0.05	0.05	0.04
0.1	0.02	0.02	0.02	0.01

TABLE 11 THE BINDING OF TIN(II) TO MOUSE EL-4 CELLS. Cells were incubated with ^{113}tin(II) in serum-free RPMI-1640 for 1 hour at 37°C. Initial binding was determined by collecting small aliquots of cells from the various treatments, on polycarbonate filters (Biorad 1um pore size) using saline washes. After the initial binding was assessed, cells were then collected by centrifugation and resuspended in tin-free medium containing 10% FCS. Binding of residual ^{113}tin(II) to cells was determined at various times after termination of the tin(II) treatment. A portion of the 113tin(II) activity remained firmly bound to cells at 24 hours after the removal of 113tin(II) from the incubation medium. The rate of loss of cell bound radioactivity was about 0.5% per hour.

Fig. 7

Effect of Tin (II) on the Rate of DNA Synthesis in EL-4 Cells

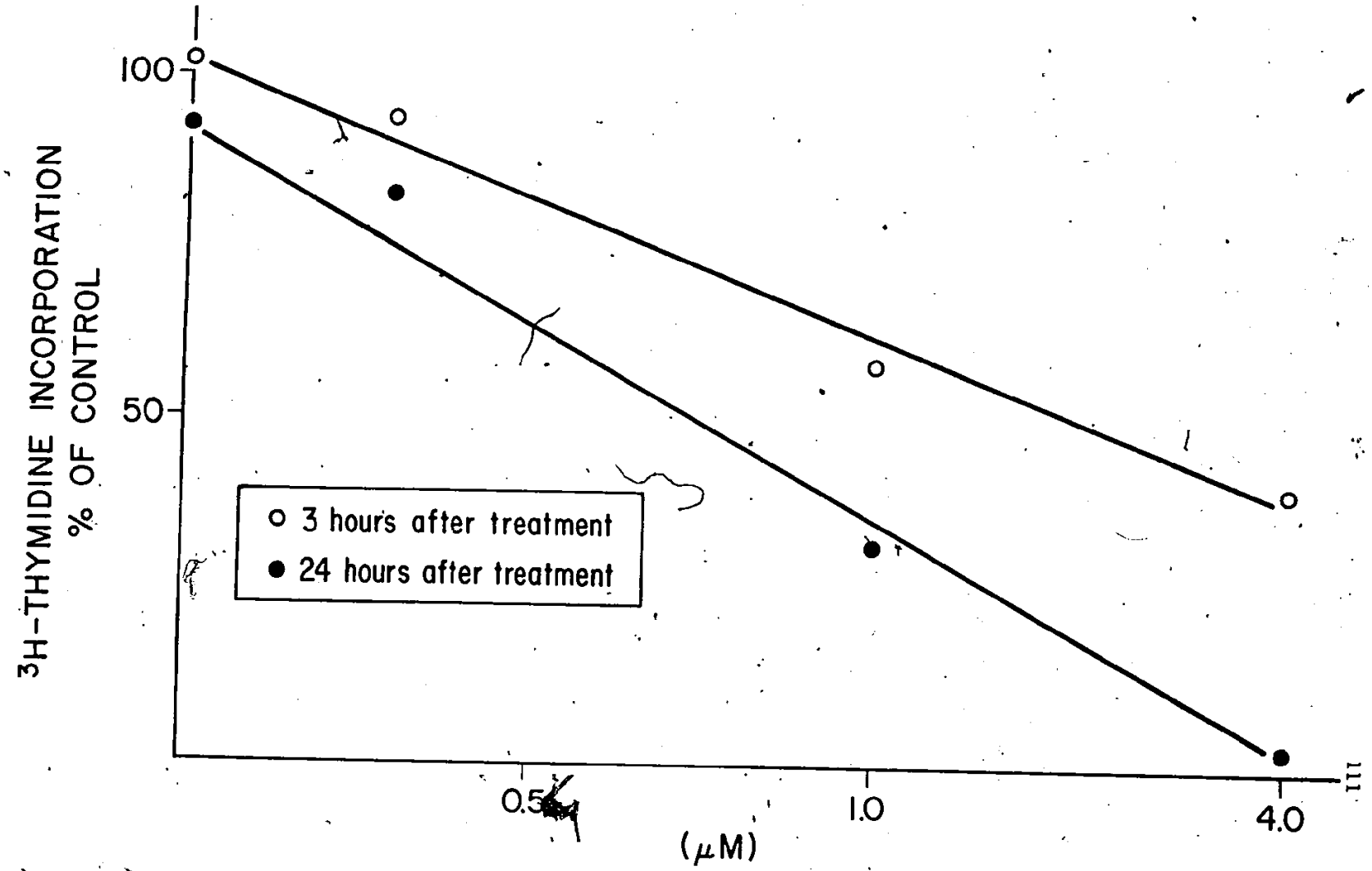


Figure 7. The dose-effect of tin(II) on the rate of DNA synthesis in mouse EL-4 cells at 3 and 24 hours after treatment. The rate of DNA synthesis decreased progressively after treatment indicating that long-lived lesions induced in G_1, G_2 and M phases prevented cells from entering or progressing through S phase.

calculated from the highest concentration on table 11.The experiments indicate that the inhibition of DNA synthesis depends on the concentration of tin(II) in the medium and on the length of the incubation time. Therefore, low concentrations of tin(II) would be able to exert a significant effect on DNA synthesis if the period of incubation were extended. This would allow time for the tin(II) to accumulate in the nucleus, the presumed sensitive intracellular target. Alternately, time would be required to generate and accumulate sufficient damage to produce an observable effect on the rate of DNA synthesis if only a small quantity of tin(II) was able to get into the nucleus. Regardless of the explanation it can be inferred from the data that tin(II) is not readily oxidized in-situ to inactive tin(IV) but would have an extended intracellular half-life. Therefore, each tin(II) taken into the nucleus would probably be able to produce more than one "hit" or lesion in the DNA and a cumulative effect can be postulated. This is especially pertinent in the light of Binder 1979 who showed that tin(II) and Fe(III) could produce a long-lived redox couple capable of generating one electron reducing equivalents.

4.2.4 Tin (II)-113 Uptake and Inhibition of DNA Synthesis in Mouse Splenocytes Stimulated for 48 Hours with Con A

This test system, which uses mouse splenocytes that have been stimulated for 48 hours with Con A, is similar to that described by Warren et al, 1980 which in turn is a modification of the DNA synthesis inhibition assay of Painter, 1977. The objective of this group of experiments was to follow suppression of DNA synthesis using autoradiography. A prompt decrease in labelled fraction and the number of large blast-like cells would indicate that tin(II) prevented the initiatiion of replicons.

DNA synthesis is stimulated in mouse splenocytes that have been incubated with Con A for 48 hours. Autoradiography of these cells, briefly pulsed (20 minutes) with ^{3}H-TdR (10uCi/ml) indicates that 17-20% are in S phase as opposed to 2-3.5% in unstimulated controls (data not shown). The treatment of these 48 hour Con A stimulated splenocytes for one hour with tin(II) in serum and Con A free medium, was sufficient to induce a prolonged suppression of DNA synthesis after cells were reincubated in tin(II) free RPMI-1640 medium supplemented with 10% FCS. Autoradiographic analysis of these cells at various times, indicated a dramatic loss in labelled fraction and also in large blast-like cells which was especially noticeable at the high tin concentrations and with increasing incubation times. This suggests that initiation of DNA replication was affected primarily, but the data is difficult to interpret beyond this simple generalization.

4.3.0 The Detection of DNA Damage In Vitro by Fluorescence Analysis of DNA Unwinding

The technique of fluorescence analysis of DNA unwinding is a rapid and flexible method for detecting DNA strand break damage in a variety of cell types that have been exposed to physical or chemical trauma. This series of experiments exploited the convenience of the FADU technique and attempted to relate a number of observations to the presence of strand break damage. Specifically, the experiments attempted to:

1. establish a relationship between tin(II) uptake, inhibition of DNA synthesis and DNA damage,
2. determine how the presence of chelating agents, which affect the cellular uptake of tin(II), could modify DNA damage,
3. determine if DNA breaks are repaired in the presence and absence of tin(II),
4. compare damage produced by tin(II) to that from known DNA damaging chemicals and gamma radiation,
5. determine if tin(II) and gamma radiation have a synergistic effect on the production of DNA damage,
6. determine if tin(II) produces DNA damage by a direct acting mechanism or by a secondary effect (DNA disassembly),
7. compare FADU and alkaline sucrose gradient analysis as methods for detecting DNA strand break damage.

4.3.1 The Detection of DNA Damage in Cells Treated With Tin(II) at 0^{o} and $37^{o}C$.

It was suggested by Painter 1977, Warren 1980 and others, that damage

to the DNA of cells is sufficient to cause a rapid and prolonged inhibition of DNA synthesis. The damaged areas on the DNA molecule act as barriers to the initiation and elongation of replication sites but the nature of these long-lived lesions is unknown. These experiments were designed to establish a relationship between DNA strand break damage and the concentration of tin(II) in the incubation medium and then to relate these parameters to the inhibition of DNA synthesis.

For convenience, the DNA damage produced in cells by a standardized chemical treatment can be defined in terms of a radiation equivalent dose. In this case, the DNA damage from a chemical treatment, is related to the dose (in Gy) of Co-60 radiation that would produce an equivalent amount of DNA damage, as measured by the FADU technique. The damage produced in the cells by the chemical treatment is then expressed in units of Gy-equivalents. Alternately, the Gy-equivalents can be converted directly into the number of strand breaks/genome. This is acceptable if the general nature of the DNA damage produced by the chemical treatment is similar to that produced by an exposure to Co 60 gamma rays. In the case of tin(II) and gamma rays, some of the DNA damage occurs directly as strand breaks or as lesions that can be converted into breaks, either enzymatically or by treatment with alkali.

The tin(II) treatment of cells suspended in BSS, produced nearly identical dose - dependent damage to DNA at $0^{o}C$ and $37^{o}C$ for concentration of tin(II) less than 10 μM, Table 12. However, DNA damage was consistently greater at $0^{o}C$ for tin(II) treatments μ10 M. The number of strand breaks/fM bound/cell increased directly with the concentration of tin(II) in the incubation medium shown in Table 13. This could indicate that initially, tin(II) had to saturate non-critical areas in the cell before binding could occur to the critical targets. Alternately, repair of DNA strand breaks could be inhibited by

TABLE 12

DAMAGE AND REPAIR IN THE DNA OF HUMAN WHITE BLOOD CELLS TREATED FOR 30 MINUTES WITH TIN(II) AT 0°C AND 37°C.

Treatment	Tin(II),fM bound/cell		Initial Damage Δ (100logD)		Repair Δ (100logD)
temperature	0	37	0	37	37
Control	-	-	-	-	-
Co-60,1Gy.			12.4	-	1.9
Tin(II,(uM)					
5	0.15	0.12	0.6	0.7	0.6
10	0.48	0.44	7.2	4.2	-1.2
25	3.11	2.71	38.3	30.1	-0.1
50	8.42	7.66	135.3	100.4	-1.9

TABLE 12. THE DETERMINATION OF DNA DAMAGE AND REPAIR, BY FADU, IN HUMAN WBC TREATED WITH TIN(II). After exposure of cells to tin(II) in BSS, initial DNA damage was determined by FADU. Repair was assessed in control and treated cells after a 20 minute repair incubation in complete medium at 37°C. DNA damage was expressed as Δ (100logD), which is proportional to the strand break damage suffered by the cells. The non-linearity of the dose-effect and the apparent threshold for damage will be discussed in table 13 and in the text.

TABLE 13.

COMPARISON OF DAMAGE PRODUCED IN THE DNA OF HUMAN WHITE BLOOD CELLS EXPOSED TO Co-60 GAMMA RAYS AND TIN(II).

Treatments	Breaks/Genome	Breaks/fM	Gy-Equivalents.
Co-60,1Gy.	1,196	-	1.000
$0^{o}C$			
Tin(II),uM			
5	45	307	0.037
10	535	1,108	0.447
25	2,845	884	2.380
50	10,051	1,262	8.404
$37^{o}C$			
Tin(II),uM			
5	52	436	0.043
10	312	707	0.261
25	2,236	825	1.900
50	7,458	979	6.236

TABLE 13. COMPARISON OF DAMAGES PRODUCED IN THE DNA OF WBC EXPOSED TO Co-60 GAMMA RAYS AND TIN(II)-THE CONCEPT OF THE Gy-EQUIVALENT DOSE. The exposure of mammalian cells to 1 Gy of Co-60 gamma rays produces 1,196 DNA strand breaks per genome, Kampf et al. 1977.The DNA damage produced from the treatment of cells with tin(II) can be expressed in terms of the dose of Co-60 gamma rays that would produce an equivalent number of strand breaks. The DNA strand break damage in cells treated with tin(II) increased rapidly in parallel with, but not as a simple linear function of the the tin(II) concentration. The non-linear response and the appearance of a threshold dose could be due to 3 factors. First, repair could be inhibited at increasing tin(II) concentrations. Second,repair pathways could be saturated by the large amount of damage produced at greater than 10 um tin(II). Third, tin(II) in dilute solution could be bound largely to the surface of the culture vessel and therefore not available to interact with the cells.

increasing concentrations of tin(II) or repair pathways could be saturated by the large amount of DNA damage produced at concentrations of tin(II) 10 M. The non-linear dose-effect and the observed threshold for DNA damage however, could also be partly artifactual. The large number of binding sites on the surface of the culture vessel could compete directly with sites on the cells for the limited quantity of tin(II) in suspension. All low concentrations, a greater proportion of the tin(II) would be bound to the more numerous sites on the container surface and the quantity available to interact with the cells would decrease at an accelerating rate. The result would be an apparent threshold for damage. This contention is supported by the observation that carrier-free ^{113}tin(II) binds strongly to glass and plastic and cannot be readily displaced by the addition of carrier tin(II). The phenomenon of surface absorption at low metal concentrations is well documented in the literature on radiochemical methods, Lavrukhina et al 1967. The observed threshold for damage therefore could be partly artifactual and would justify the use of extrapolated doses at low tin(II) concentrations in-vitro.

The detection of DNA damage at 0^{o}C, as well as 37^{o}C, strengthens the evidence supporting the earlier contention that the uptake of tin(II) does not require the expenditure of cellular energy. The observation that tin(II) produces DNA damage at 0^{o}C also tentatively suggests that tin(II) acts directly on DNA rather than through some secondary effect, such as DNA disassembly, which is an energy dependent process (Williams et al, 1974).

4.3.2 The Detection of DNA Damage in Whole Cells Treated With Tin(II) Chelates at 0 C and 37 C

The presence of various tin(II) complexing agents in the incubation medium can modify the uptake of ^{113}tin(II) by human WBC suspended in BSS. The

TABLE 14

Gy-EQUIVALENT DNA DAMAGE IN HUMAN WBC TREATED

BY TIN(II) CHELATES AT 37°C.,AS MEASURED BY FADU.

Treatment	Damage (100log D)	Tin(II) fM/Cell	Breaks/ Genome	fM	Gy-Equivalent
Co-60,1 Gy	12.4		—		1.000
Tin(II),50uM as;					
Chloride	100.4	7.66	7,458	974	6.236
CDTA	62.7	1.15	4,658	3,517	3.894
Citrate	100.9	n.d.	7,469	n.d.	6.280
Phytate	97.4	16.75	7,235	26	6.049
Colloid(F^-	28.2	3.42	2,094	179	1.751
Phosphate	100.9	n.d.	7,428	n.d.	6.211
MDP	25.1	0.18	1,865	57,706	1.559
Pyp	0.8	2.38	59	10	0.049
Hedspa	0.6	n.d.	45	n.d	0.037
EDTA	1.6	0.02	119		0.099
MAA	15.6	n.d.	1,159	n.d.	0.970
HSA	0.8	0.13	59	3,537	0.050

TABLES 14-15. THE Gy-EQUIVALENT DNA DAMAGE PRODUCED IN HUMAN WBC BY TREATMENT WITH TIN(II) CHELATES AT 37°C AND 0°C AS MEASURED BY FADU. The tin(II) chelates of CDTA and MDP produced the largest number of DNA strand breaks/fM of tin(II) bound per cell at 37° C. Table 15 shows a similar set of treatments done a 0° C.The large number of breaks/fM calculated for tin(II) EDTA is artifactual due to a small, but measureable, uptake of the complex.

TABLE 15

Gy-EQUIVALENT DNA DAMAGE IN HUMAN WBC TREATED BY TIN(II) CHELATES AT 0°C, AS MEASURED BY FADU.

Treatment.	Damage Δ(100logD)	Tin(II) fM/Cell	Breaks/ Genome	fM	Gy-Equivalents.
Co-60,1Gy.	12.4		1,196		1.000
Tin(II),50uM as;					
Chloride	135.3	8.42	10,051	150	8.404
CDTA	76.7	2.37	5,698	1,015	4.764
Citrate	100.5	n.d.	7,466	n.d.	6.242
Phytate	98.6	15.49	7,325	30	6.124
Colloid					
(F-)	36.1	0.70	2,682	5,440	2.242
Phosphate	105.0	n.d.	7,800	n.d.	6.584
MDP	0.5	0.03	37	-	0.030
PyP	0.5	0.70	37	84	0.030
Hedspa	0.6	n.d.	45	n.d.	0.037
EDTA	2.2	0.02	163	58	0.137
MAA	33.1	n.d.	2,459	n.d.	2.056
HSA	0.5	0.13	37	-	0.137

*See table 14 for discussion.

uptake of these tin(II) complexes is related to their ability to produce DNA damage by this group of experiments. In addition, however, most of these chelating agents are used in diagnostic nuclear medicine and so it is important to determine if their tin(II) complexes also produced DNA damage.

The tin(II) chelates of CDTA, citrate, phosphate, fluoride, chloride and phytate produced damage in the DNA of cells treated at $0^{o}C$ and $37^{o}C$, Tables 14 and 15, but DNA damage was consistently higher when treatments were done at $0^{o}C$. All of these tin(II) complexes taken up by the cells at both temperatures, Table 8. Tin(II) MDP produced damage at $37^{o}C$ but not at $0^{o}C$ which reflects the cellular uptake of this complex. Tin(II) chelates of EDTA, pyrophosphate, Hedspa and human serum albumin did not produce damage at either temperature after 30 minutes of incubation in BSS. DNA damage was not found in the cells of control cultures that were treated with only the ligands (data not shown). Incubation of human WBC with 100 μM tin(II), in the presence of 6% autologous human serum, produced detectable damage after 12 hours (data not shown).

Tin(II) pyrophosphate was taken up by the cell at both temperatures but did not produce any DNA damage. It is possible for each tin(II) to bind 2 molecules of pyrophosphate. In this case there would be no free electrons available to participate in chemical reactions, which could account for the lack of DNA damaging properties of this complex.

The amount of DNA damage produced by various chelates of tin(II) therefore, appears to depend on the amount that is taken up by cells and on the state of the electron orbitals in the complex. Tin(II) chloride produced the highest Gy-equivalent damage of all the chelates tested, Table 14, by virtue of its massive uptake (8.42 fM/cell at $0^{o}C$ and 7.66 fM/cell at $37^{o}C$). The number of breaks generated/fM of tin(II) bound was 1262 at $0^{o}C$ and 979 at $37^{o}C$. The compounds that were most efficient at producing DNA damage however were

TABLE 16.

THE EFFECT OF TIN(II), TIN(IV) AND MERCURY(II)

ON CELL VIABILITY AND FADU RESULTS.

Treatment	Cell Viabilty	Damage Δ(100logD)
Control	100	-
Tin(II)	100	97.4
Tin(IV)	100	1.5
Mercury(II)	0	110.9

TABLE 16. THE EFFECT OF TIN(II) AND (IV) AND MERCURY(II) ON CELL VIABILITY AND FADU RESULTS. Human WBC at 2x106 per ml were treated for 30 minutes in BSS at 37oC with the metals at a concentration of 50uM. Cell viability was assessed using the trypan blue exclusion test. Mercury(II) treated cells were all dead after the incubation and the observed strand breaks were presumeably the result of DNA disassembly,Williams et al.1974.This suggests that dead cells could be a potential source of false positives for the FADU assay and indicates that the range of treatments should be examined to establish a viability index before proceeding to assess DNA damage using the FADU method.

Sn(II)-CDTA and Sn(II)-MDP which produced 4,045 and 10,389 breaks/fM/cell respectively, at 37°C, Table 14.

4.3.3 How Does Tin(II) Damage DNA?

Primary and Secondary Mechanisms.

The mechanism by which tin(II) produces DNA damage is of considerable interest to this study. Two general hypotheses can be envisaged to explain the DNA damage observed in cells exposed to tin(II). A primary or direct mechanism requires that tin, or a chemically produced reaction product (such as a free radical), interact directly with the DNA strand to produce a frank break or damage that can be converted to a break by alkali treatment. A secondary mechanism involves enzymatic degradation of DNA, such as DNA disassembly, Williams et al, 1974, 1976, which is a response of the cell to some non-DNA damaging trauma or DNA damage that is incurred when phagocytic cells, in response to a membrane-particle interaction, release a burst of oxidizing activity. The free radicals produced during this "burst" are capable of producing not only self-inflicted DNA damage but also damage to other cell populations in the incubation mixture.

4.3.3.1 The Detection of Direct DNA Damage by Tin(II) in Cell Lysates

Cells which are gently lysed at 0°C in a urea solution release high molecular weight DNA. The addition of tin(II) directly to this lysate produced DNA damage which was about 60% of the damage produced by treatment of whole cells in BSS at 300 μM (data not shown). This simple test indicates tin(II) was able to produce damage directly in DNA and at 0°C indicating that the mechanism was not enzymatic or energy dependent. It could not be determined if tin(II) or some

related reactive species, attacked the DNA phosphate backbone or the DNA bases or both. The fact that there is a prolonged suppression of DNA synthesis in tin(II) treated cells however, suggests that some form of long-lived perturbation remains in the conformation of the DNA.

4.3.3.2 DNA Disassembly: The Effect of Cell Death on the Observation of DNA Strand Breaks by FADU

Treatments that are lethal or cytotoxic can increase the number of DNA strand breaks that are observed by FADU but these are related to DNA disassembly from cell death rather than to primary DNA damage. Cytotoxicity, therefore is one possible source of false positives for this assay.

DNA damage produced in cells by tin(II), tin(IV) and mercury(II) after incubation at 37°C is shown in Table 16. Tin(IV) produced neither DNA damage nor loss in cell viability; tin(II) produced DNA damage without loss in cell viability and mercury(II) produced total loss in cell viability and total degradation of DNA. A prudent FADU protocol therefore requires that the viability of cells be determined at a number of concentrations of the agent being tested. The maximum concentration of the test agent that should be used in the FADU procedure should produce no more than a 10% loss in cell viability after 45-60 minutes of incubation in BSS at 37°C. This criterion was used throughout this thesis and is arbitrary since no attempt was made to correlate the proportion of dead cells to FADU results.

4.3.3.3 The Effect of 2-Deoxyglucose on DNA Damage From Treatment of Cells with Tin(II) Colloids

Phagocytic cells can release an "oxidative burst" when they interact with particles or when their membranes are perturbed by surface active agents

such as deoxycholate (Tsan et al, 1980) or phorbol myristate acetate. This "oxidative burst", which is autotoxic and cytotoxic to the surrounding cells, can be prevented by incubation at $0^{o}C$ or in the presence of antimetabolites, such as 2-deoxyglucose, (2-DOG), which inhibits oxidative phosphorylation.

The preincubation of cells with 2-DOG did not prevent colloids of tin(II) or tin(II) phytate from producing DNA damage at either $0^{o}C$ or $37^{o}C$, Table 17. This indicates that colloids of tin(II) did not trigger the "oxidative burst" and therefore DNA damage probably did not arise by a secondary (indirect) mechanism.

4.3.4 The Effect of EDTA on DNA Damage Produced in Human WBC by Treatment with Tin(II)

This experiment was designed to determine how quickly tin(II) produced DNA damage in cells. The rationale was that the addition of 1 mM EDTA to the incubation mixture at various times would prevent further uptake of tin(II) and thus prevent further damage. It would not however, prevent the accumulation of DNA damage from the action of tin(II) that had been taken into the cells before the addition of EDTA to the incubation medium.

Treatment of cells with 50 μM tin(II) for 15 minutes before the addition of 1 mM EDTA to the incubation mixture, rapidly produced the expected level of DNA damage (data not shown). This indicates that the production of DNA damage reflects the kinetics of tin(II) uptake. When the incubation was allowed to continue for 20 minutes in the presence of EDTA, the level of damage remained unchanged. Therefore, the effect of the intracellular tin(II) was prolonged either because repair of strand break damage was inhibited or because the tin(II) remained reactive in-situ with each molecule being capable of generating a number of "hits" or lesions into the DNA.

TABLE 17

THE EFFECT OF 2-DEOXYGLUCOSE (2-DOG) ON DNA DAMAGE IN HUMAN WBC TREATED WITH TIN(II) COLLOID PREPARATIONS.

Treatment	DNA DAMAGE - Δ(100logD).	
Tin(II),uM	0oC.	37oC
Control	-	-
Tin(II) Phytate	187.2	183.8
Tin(II) Phytate plus 2-DOG	187.2	180.0
Tin(II) Chloride	140.9	109.0
Tin(II) Chloride plus 2-DOG	135.3	100.4

TABLE 17. THE EFFECT OF 2-DOG ON DNA DAMAGE RESULTING FROM THE TREATMENT OF CELLS WITH COLLOIDAL TIN(II). The presence of 2-DOG can prevent phagocytic cells from releasing a burst of oxidizing power into the medium in response to stimulation by colloids or particles. The oxidizing capacity can be autotoxic and cytotoxic to the surrounding non-phagocytic cells. The observed damage could therefore be due to a secondary rather than to a primary or direct effect on the DNA of the cell. Tin(II) chloride and tin(II) phytate are colloidal in aqueous media and therefore they could potentially stimulate phagocytes. The presence of 2-DOG in the incubation medium didnot prevent either tin(II) chloride or phytate from producing DNA damage suggesting a primary mechanism could be responsible for the damaging effects of tin(II). The increased DNA damage observed in cells treated with tin(II) in the presence of 2-DOG could be due to an inhibition of their repair capacity, a metabolic process which is known to be energy dependent.

4.3.5 DNA Damage Produced in Human WBC From Combined Treatment with Tin(II) and Co-60 Gamma Rays

One aspect in the study of DNA damaging agents is to determine how they interact with other treatments, which may or may not produce DNA damage. The biological interaction of tin(II) with gamma radiation is of particular importance because tin(II) is almost always present in radiopharmaceuticals that are labelled with ^{99m}Tc, a radionuclide that emits gamma rays at 140 KeV. Patients undergoing diagnostic nuclear medicine procedures, therefore, receive simultaneous exposures from two DNA damaging agents. This experiment measures the total DNA strand break damage in cells that have been exposed simultaneously to tin(II) and Co-60 gamma ray treatments and attempts to assess the effect of tin(II) on the repair of the total strand break damage.

The exposure of human WBC to 25 μM tin(II) for 15 minutes at 37°C in BSS, followed by irradiation at 0°C with 2 Gy of Co-60 gamma (0.1556 Gy/minute), produced more damage than either treatment alone, Table 18. This indicates that the two together were synergistic for DNA damage. The repair of DNA strand breaks appears to be inhibited by the presence of tin(II) but this result could also reflect the saturation of the repair process which would occur if the damage to the DNA was extensive.

4.3.6 Validation of the Fluorescence Analysis of DNA Unwinding

Validation of Technique In Vitro

The effect on DNA of cells treated with a variety of agents is shown in Tables 19 and 20. Some of these agents are known to damage DNA while others are not damaging under the assay conditions. For all treatments, the cells remained greater than 88% viable by trypan blue exclusion. The results were compiled into

TABLE 18

THE EFFECT OF TIN(II) TREATMENT AND COBALT-60 GAMMA RADIATION ON DNA DAMAGE IN HUMAN WHITE BLOOD CELLS.

Treatment	Observed Damage Δ (100logD) Initial	Repair	Breaks/Genome. Initial	Repair
Tin(II) 25 uM	31.3	0.1	3110	8
Co-60 2 Gy	22.5	3.0	2237	240
Tin(II) and Co-60	120.6	17.4	12400	1,675

Table 18. THE SYNERGISTIC EFFECT OF TIN(II) AND GAMMA RAYS ON DNA DAMAGE IN HUMAN WBC. Cells were treated initially with 25uM tin(II) in BSS for 15-20 minutes, placed on ice and then irradiated with Co-60 gamma rays. Some cells were assayed immediately for DNA damage by FADU. Other cells were washed 3 times in ice cold medium and then incubated for for 20 minutes in the presence of 5% autologous serum to measure repair. The DNA damage resulting from the combined treatments was greater than the damage resulting from each treatment alone. The residual damage after the repair incubation could be due to the inhibition of repair by tin(II) or, more likely, from saturation of the repair pathway caused by the massive DNA damage of the combined treatments. Tin(II) would be present in the target organs of patients simultaneously with mCi quantities of the gamma emitting radionuclide Tc-99m (140KeV) during the administration of diagnostic radiopharmaceuticals. The combined effects of these could therefore underestimate the dose to the patient by a factor of about 3-4, if extrapolation can be adequately made into the low dose range.

TABLE 19

DAMAGE TO DNA, MEASURED BY FADU, FROM EXPOSURE OF HUMAN WBC TO VARIOUS AGENTS.

Agent	Concentration	DNA Damage Δ (100logD)	Gy-Equivalent
Control			0
1.Known DNA damagers			
Bleomycin	0.5ug/ml	2.1	0.2
	5.0ug/ml	18.9	1.4
	50.0ug/ml	88.7	6.4
Ethylmethane-sulfonate	1.2x10-5M	-0.2	
	1.2x10-4M	0.9	0
	1.2x10-3M	9.6	0.8
	1.2x10-2M	92.7	6.7
4-Nitroquinoline-N-Oxide	2.0x10-8M	-0.8	0
	2.0x10-7M	13.2	1.1
	2.0x10-6M	89.1	6.4
	2.0x10-5M	119.6	8.6
2.Presumed non-damagers.			
Cyclophosphamide	1.0x10-5M	-2.1	0
	1.0x10-4M	-1.8	0
	1.0x10-3M	-1.7	0

TABLE 19. THE EFFECT OF VARIOUS AGENTS ON DNA DAMAGE IN HUMAN WBC AS MEASURED BY FADU. Various agents were tested for their ability to produce DNA damage under the assay conditions employed by FADU. Agents that are known to introduce damage directly into the DNA of cells were positive by FADU. Cyclophosphamide, which must be activated to the DNA damaging species by enzymatic processing, was negative in the FADU procedure.

TABLE 20

DAMAGE TO DNA FROM EXPOSURE OF HUMAN WBC TO VARIOUS CARCINOGENIC AND NON-CARCINOGENIC METALS.

Treatment 50uM,Metal	Observed Damage Δ(100logD)	Gy-Equivalents.
Control	-	0
UO_2(II)	3.1	0.2
Cr(VI)	9.4	0.8
Cr(III)	1.3	0
Co(II)	17.7	1.5
As(III)	-2.7	0
Zn(II)	0.6	0
Cd(II)	1.5	0
Pb(II)	-0.1	0
Mn(II)	0.1	0
Ni(II)	2.1	0.1
Sb(III)	1.1	0
VO_2	0.5	0
Sn(IV)	0.7	0
Sn(II)	104.4	7.0

TABLE 20. THE EFFECT ON THE DNA OF HUMAN WBC TREATED BY HEAVY METALS. Cells at $2X10^6$/ml in BSS were treated at 37oC.for 30 minutes with the metals at 50uM, before being assessed for DNA damage by FADU. Sn(II) was found to be the most damaging of all the metals tested being about 10 times more damaging than Cr(VI), a known human carcinogen , Costa 1980. Uranyl, (UO_2) and nickel (Ni(II)), were borderline for damage Arsenic(III), a known human carcinogen was negative by FADU.

a 2x2 contingency table and the data from FADU and the literature were tested for goodness of fit by the Chi square procedure. The hypothesis tested is that the proportion of agents that give false positives and false negatives by FADU is the same as the proportion that give true positives and negatives for DNA damage. Using the procedure of Armitage, 1971, the Chi square = 13.162 for 3 degrees of freedom. This means that the proportion of true positives and negatives is significantly different from the proportion of false positives and negatives at $p = 0.005$. The correlation coefficient, $r = .81$, indicates that there is a reasonably strong association between the results from FADU and the literature. The sensitivity of FADU for detecting DNA damaging agents is 78%, which is the proportion of known DNA damaging agents that are positive by FADU. No false positives were found in the limited number of substances tested.

4.3.7 The Effect of Tin(II) and Tin(IV) Treatment on the DNA of Chinese Hamster Ovary Cells as Analyzed on Alkaline Sucrose Gradients

DNA strand break damage, produced by treatment of CHO cells with tin(II), tin(IV) and Cr(VI), was assessed by the well established technique of alkaline sucrose gradients. This method was used to confirm the results obtained by the FADU technique which used similarly treated human WBC.

Cells prelabelled with ^{14}C-TdR were used as solvent controls while those prelabelled with ^{3}H-TdR were treated with tin(II), tin(IV) or Cr(VI) for 60 minutes at 37^{o}C in serum-free medium. Control and metal treated cells were added sequentially to the lysis layer on top of the alkaline sucrose gradient and then processed as previously described. The calculation of the molecular weight profiles was carried out using the method of Palcic et al, 1972. Cell survival was done in parallel with the alkaline sucrose gradient analysis by estimating

TABLE 21

THE DETECTION OF DNA STRAND BREAKS BY ALKALINE SUCROSE GRADIENT ANALYSIS IN CHINESE HAMSTER OVARY CELLS CELLS TREATED WITH TIN(II), TIN(IV) AND CHROMIUM(VI).

Treatments.	Breaks/ 10^8D.	Breaks/ Genome	Gy-Equivalents.
Tin(II),uM			
50	0.18	6,480	5.20
150	0.35	12,600	10.10
350	0.66	23,760	19.00
550	0.80	28,800	23.00
Tin(IV),uM			
100	0.0009	32	0.03
Cr(VI),mM			
10	0.22	7,920	6.34
Co-60,Gy			
1	0.035	1,196	1.00

TABLE 21. THE USE OF ALKALINE SUCROSE GRADIENT ANALYSIS TO DETECT DNA DAMAGE IN CHO CELLS TREATED WITH TIN. Alkaline sucrose gradient analysis was used to confirm the DNA damaging properties of tin(II) which were observed by the FADU technique using human WBC. The number of strand breaks/genome observed at 50uM tin(II) by alkaline sucrose gradients was similar to the 7,458 (table 13) observed by FADU. A 7 day survival study was done on the tin(II) and tin(IV) treated cells to supplement the data obtained by alkaline sucrose gradient analysis on strand break damage. There was no loss in cell survival after 7 days, at any of the concentrations examined, however the colonies were not comparable either in size or in capacity for stain. This indicates that all of the DNA damage was repaired sufficiently in CHO cells to allow DNA to replicate. This could indicate that CHO cells have a greater capacity than lymphoid cells to repair DNA damage.

relative colony forming ability after treatments.

The results of alkaline sucrose gradient analysis for tin(IV), tin(II) and Cr(VI) are shown in Figures 8, 9 and 10 respectively. Tin(IV), at the highest concentration tested (550 μM) did not cause any perceptible DNA damage. Tin(II) treatment at 550 μM however caused extensive DNA damage as indicated by the shift or displacement of the treatment (^{3}H) peak towards lower molecular weight. The Cr(VI) treatment at 10 mM was used as a positive control.

The effect of tin(II) on the DNA of CHO cells is summarized in Table 19. The treatment of CHO cells with tin(II) at 50 μM produced 6,480 strand breaks/genome, which is equivalent to 5.2 Gy. This is comparable to the 7,458 breaks/genome (6.2 Gy) found for tin(II) at 50 μM by the FADU technique.

The colony forming ability of CHO cells was not affected by either tin treatments at concentrations up to 1 mM. CHO cells rapidly recovered their ability to synthesize DNA and undergo cell division after tin(II) treatment, Table 19. This contrasts sharply with mouse and human lymphoid cells which undergo a dramatic loss in their ability to synthesize DNA after tin(II) treatments, Table 10, Figures 3, 4 and 5. The quantity of tin(II) required to induce observable DNA damage in CHO cells by alkaline sucrose gradient analysis is 10 times that required to induce detectable DNA damage in human WBC by FADU. This indicates that CHO oells may have a greater capacity than lymphoid cells to repair the lesions induced in DNA by tin(II).

4.4.0 The Effect of Tin(II) on the Immune System of Mice

The next stage in the investigation was to determine if the effect on DNA synthesis observed after tin(II) treatment of cells *in vitro* had any consequence *in vivo*. An agent that induces a prolonged suppression of DNA synthesis and blastogenesis has the potential to be immunosuppressive in the

Figure 8. The effect of tin(IV) on chinese hamster ovary cells. Cells, labelled with ^{3}H-thymidine, were treated with tin(IV), mixed with ^{14}C-thymidine labelled solvent treated cells, lysed and centrifuged on alkaline sucrose gradients. Strand breaks were not detected in the cells after tin(IV) treatment and there was no loss in cell survival as measured by colony forming ability.

FIGURE 8

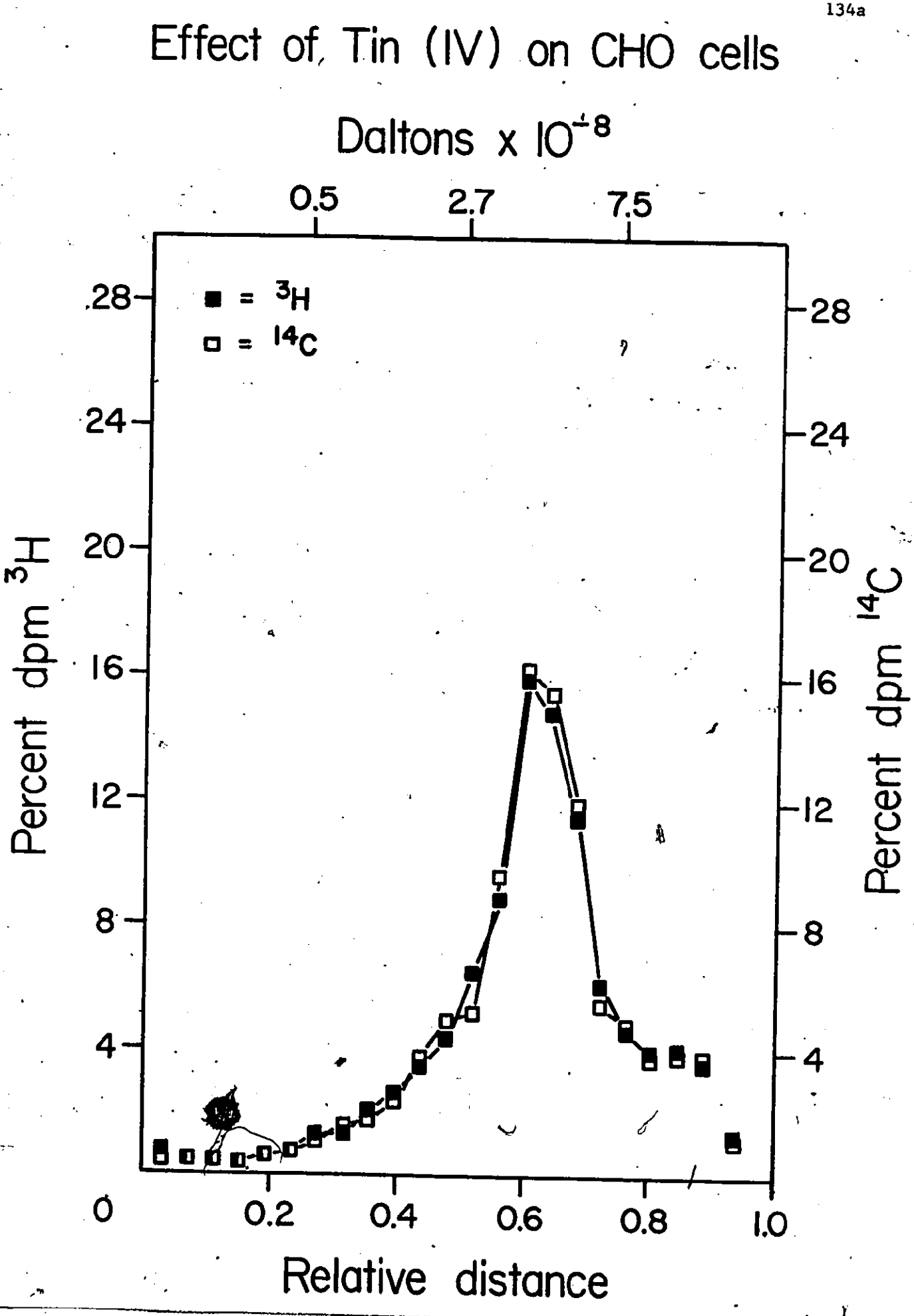
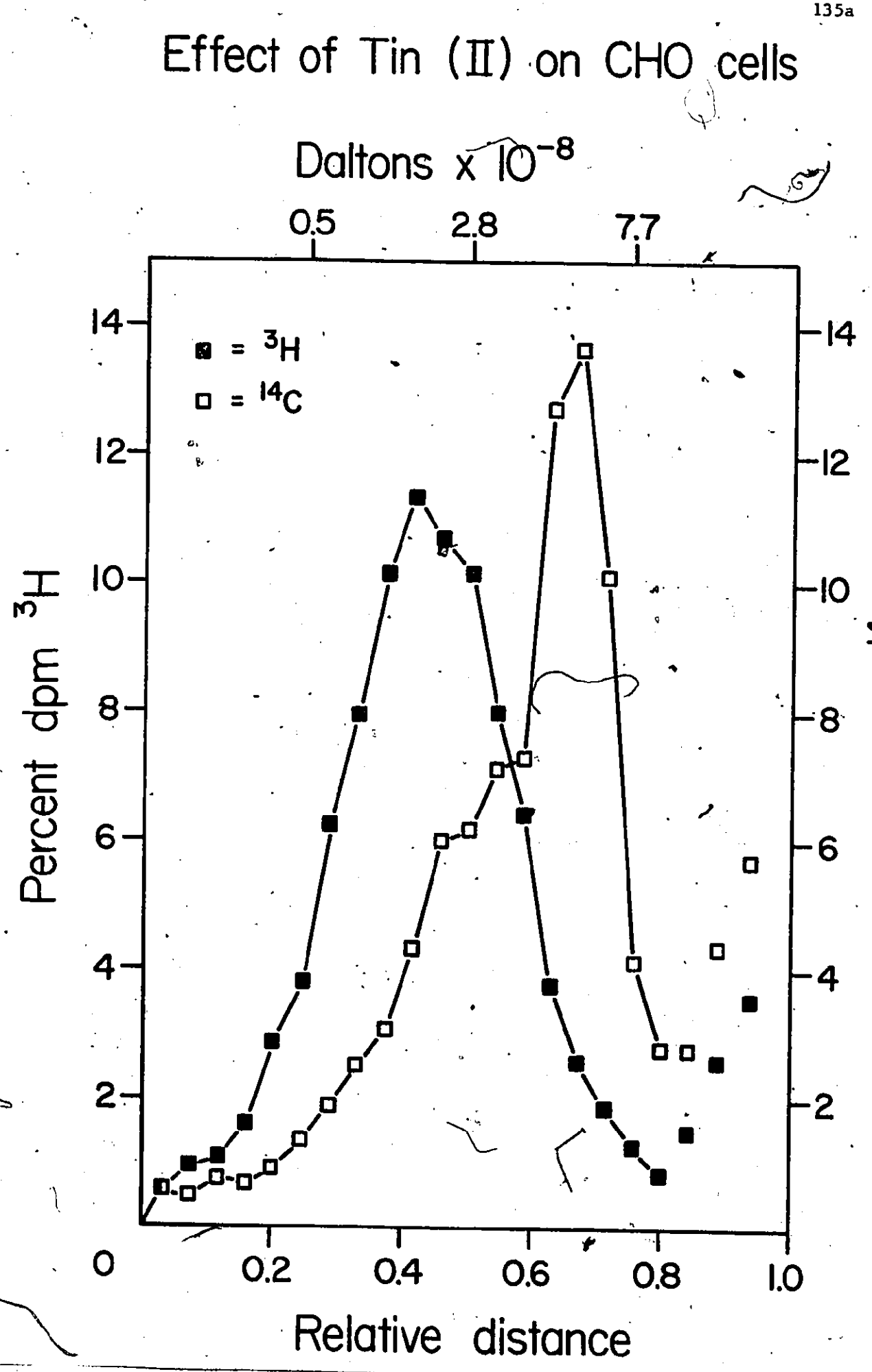

Effect of Tin (IV) on CHO cells
Daltons x 10^{-8}
0.5
2.7
7.5
■ = ^{3}H
□ = ^{14}C
Percent dpm ^{3}H
Percent dpm ^{14}C
28
24
20
16
12
8
4
0
0.2
0.4
0.6
0.8
1.0
Relative distance

Figure 9. The effect of tin(II) on Chinese Hamster Ovary cells. The shift of the ^{3}H-labelled peak towards a lower molecular weight indicated that the treatment of cells with tin(II) at 550 uM produced extensive DNA damage.

FIGURE 9.


Effect of Tin (II) on CHO cells
Daltons x 10^{-8}
0.5
2.8
7.7
■ = ^{3}H
□ = ^{14}C
Percent dpm ^{3}H
Percent dpm ^{14}C
0
2
4
6
8
10
12
14
0.2
0.4
0.6
0.8
1.0
Relative distance

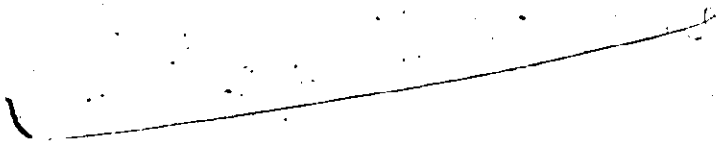
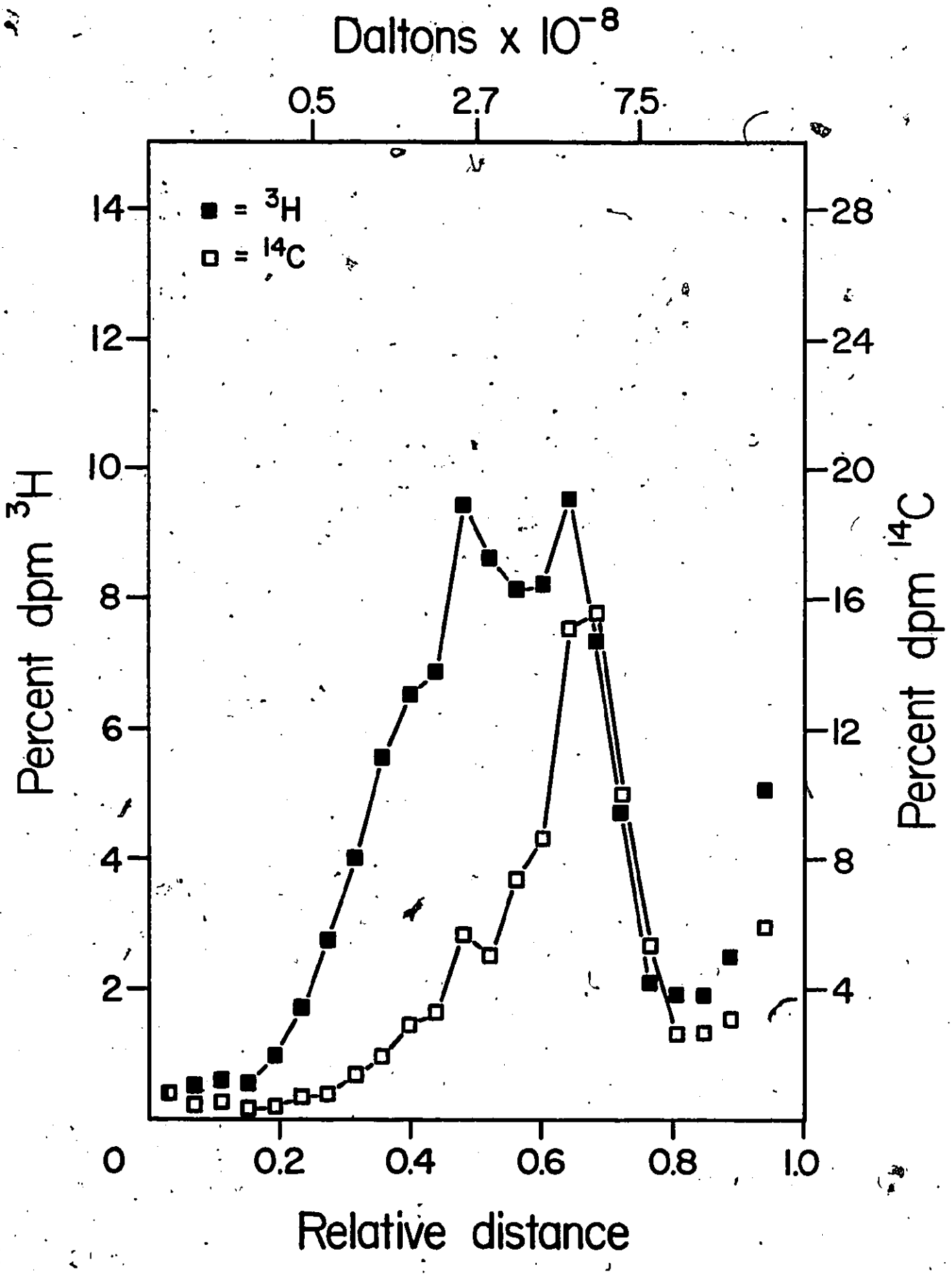


Figure IO. The effect of Chromium(VI) on chinese hamster ovary cells. Cr(VI) was used as the positive control for DNA damage in the tin(II). experiments. The treatment of cells with IOmM Cr(VI) produced about 7920 strand breaks per genome which is equivalent to that produced by 6I uM tin(II). On a molar basis therefore, tin(II) is about I64 times more potent than Cr(VI) , a known human carcinogen, at producing DNA strand break damage.

FIGURE 10

Effect of Cr (VI) on CHO cells



intact animal. The C57B6/10 mice, used in this study, have a selectively inbred sensitivity to the effect of toxins and is widely used in Federal government laboratories for toxicity testing (Dr. J. Wong, Animal Resources Center, private communications). This strain was used to define the effect of tin(II) on the immune system in vivo and to determine the biokinetics of tin(II)) as it relates to the observed biological effects.

4.4.1 The Effect of Tin(II) on the Primary Immune Response In Vivo

The primary immune response measures the capacity of the to produce primary antibodies (IgM) to an antigenic challenge by sheep red blood cells (SRBC). Briefly, SRBC were administered, by intraperitoneal injection, to mice two days after being treated by injections of tin(II). Five days after SRBC were administered, the mice were sacrificed and their spleen cells assayed for specific antibody forming cells by a complement mediated plaque assay (Cunningham et al). The results are presented in Table 22 indicate that a graded inhibition of plaque formation was produced by increasing doses of tin(II). Values from each treatment were compared within experiments and then normalized and combined. Means were compared by the Duncan's multiple range test at $p = 0.01$ and all were statistically different from control when the normalized values were compared. This means that tin(II) was able to exert an inhibitory effect on the immune system in vivo probably by suppressing DNA synthesis and blastogenesis in the lymphoid cells. The possibility that the immunosuppression is caused by a general or selective cytotoxic effect on lymphoid tissues however, could not be ruled out.

Very recently, Lawrence 1981, has shown that very dilute solutions of tin(II), 1 μM, is capable of producing a statistically significant inhibition of plaque forming cells by an in-vitro technique (Mishell et al, 1967). This adds.

TABLE 22

THE EFFECT OF TIN(II) ON THE PRODUCTION OF PLAQUE-FORMING CELLS (PFC) IN MOUSE SPLEENS.

Dose Injected mg kg-1	PFC/2x106 (mean ±sd) Spleen Cells	% Control.
Control	3,184 ±102	100
0.6	2,716 ±107	85
1.0	1,520 ±115	48
3.0	1,340 ±105	42
5.0	701± 100	22

TABLE 22. THE EFFECT OF TIN(II) TREATMENTS ON THE PRODUCTION OF ANTIBODY FORMING CELLS IN MOUSE SPLEEN. Mice were given a single intraperitoneal injection of tin(II) 2 days prior to the administration of SRBC. Five days after this injection mice were sacrificed and their spleens were asseesed for the prescence of antibody-forming cells. The injections of tin(II) produced a statistically significant (p=0.01) inhibition of antibody formation. All experiments were repeated 3 times with similar results.

credibility to the findings reported in this thesis, for immunosuppression after in vivo treatments.

4.4.2 The Effect of Tin(II) on Mixed Lymphocyte Reaction (MLR)

The MLR is another facet of the immune system and measures the ability of spleen cells to undergo blastogenesis in response to foreign cells. In this case, the spleens of mice treated with tin(II) were removed and made into single cell suspensions, enriched for T-cells by treatment with wheat germ agglutinin and then used as responders for a one-way MLR. Spleen cells, isolated from Swiss-Webster and Balb.C mice were used as stimulators in the MLR after being treated in vitro with mitomycin.C. The results, shown in Table 23 indicate that some inhibition in the response of cells from tin(II) treated animals occurred even at the lowest dose tested. The treatment of stimulator cells with tin(II) did not affect the MLR (data not shown).

4.4.3 The Effect of Tin(II) on Incorporation of ^{3}H-Thymidine Into Spleen and Bone Marrow Cells In Vivo

The effect of tin(II) on DNA synthesis has been documented after a brief treatment of cells in vitro. The exposure of cells to tin(II) in vivo however is different in that the target tissues in the animal are exposed to lower concentrations of tin(II) for longer periods of time. The ability of tin(II) to inhibit DNA synthesis in vivo may explain why tin(II) is immunosuppressive but the mechanism of this immunosuppression could also be due to cell death.

The effects of single tin(II) intraperitoneal injections on DNA synthesis in vivo are shown in Table 24. The injections caused a concentration-dependent inhibition of DNA synthesis in spleen and bone marrow.

TABLE 23

THE INHIBITION OF ONE-WAY MIXED LYMPHOCYTE REACTION (MLR) IN MURINE SPLENOCYTES PRETREATED IN VIVO FOR 2 DAYS WITH TIN(II).

Treatment	^{3}H-Thymidine Incorporation	% Control	Stimulation Index.
1. a_0b_mm	14,125±1453	100.0	8.9
2. a_1b_mm	12,592 1120	89.0	7.9
3. a_3b_mm	8,095 160	57.3	5.1
4. a_5b_mm	6,619 423	47.0	4.2
5. aam	1,585 202	-	-

TABLE 23. THE EFFECT OF TIN(II) TREATMENTS ON ONE-WAY MLR IN MOUSE SPLEEN CELLS. Responder cells were isolated from the spleens of C57B6/10 mice which had been treated with single injections of tin(II) at n=0,1,3 and 5 mgkg-1 for 2 days prior to sacrifice. These cells (a_n) were enriched for T-cells and then co-cultured, in a one-way MLR, with mitomycin C inactivated stimulator cells (b_m) isolated from the spleens of balb C and Swiss-Webster mice. The ability of the responders(a_n) to undergo blastogenesis as a result of interaction with stimulator cells(b_m) was inhibited at all of the tin(II) concentrations studied.

TABLE 24

THE EFFECT OF TIN(II) ON THE INCORPORATION OF ^{3}H-THYMIDINE INTO THE BONE MARROW AND SPLEEN CELLS OF MICE WHICH HAVE BEEN TREATED WITH SHEEP RED BLOOD CELL (SRBC) ANTIGEN.

Tin(II) Injection mg/kg	CPM/10^{6} Cells (% Of Controls.) SPLEEN	BONE MARROW
Control	4,284±1,065	7,431±248
0	9,287 130 (100.0)	11,723 1,336 (100.0)
0.3	8,235 270 (88.7)	8,794 236 (75.0)
0.6	8,660 195 (93.2)	n.d.
1.0	6,352 136 (68.4)	7,080 330 (60.3)
5.0	3,650 370 (39.0)	n.d.

TABLE 24 THE INHIBITION OF ^{3}H-THYMIDINE INCORPORATION IN BONE MARROW AND SPLEEN CELLS OF MICE THAT HAVE BEEN TREATED WITH SRBC ANTIGEN. Mice were given intraperitoneal injections of tin(II) 2 days before being treated with SRBC antigens. Five days after SRBC were administered, mice were sacrificed, bone marrow and spleen cells were isolated and cultured for 1 hour in medium containing ^{3}H-thymidine (10uCi/ml) and then the incorporation of radioactivity into the cells was assessed. All of the tin(II) treatments suppressed DNA synthesis with doses 1 mgkg^{-1} being statistically significant at $p=0.05$. In some experiments, cells were labelled *in vivo* by giving injections of ^{3}H-thymidine (2 uCi/g body weight) 1 hour before sacrifice with similar results being obtained (data not shown). This indicates that tin(II) treatments have the potential to suppress blastogenesis and ultimately the immune response in mice.

About 1 mg kg^{-1} was needed to inhibit the rate of DNA synthesis by 50% at 5 days after tin(II) injection. The difference between all treatment values and controls were statistically significant. Injections of tin(IV) at 2 and 4 mg kg^{-1} did not produce any inhibition in splenocytes at 48 hours (data not shown because of the small sample size). The acute lethal dose of tin(II) by intraperitoneal injection is about 25 mg kg^{-1} in 20 g C57B6/10 mice. The possibility that the effects on DNA synthesis is due to cytotoxicity and the selective elimination of the responding population cannot be ruled out but few dead cells, by trypan blue exclusion, were detected in the spleens of mice 5 days after sensitization with SRBC. Tin also inhibited DNA synthesis in thymus cells (data not shown).

4.5.0 The Biokinetics of Tin(II) in Mice

Biokinetics refers to the parameters that describe the tissue distribution, retention and metabolic fate of tin(II) in mice.

The tissue distribution is important to determine which organ systems could be affected by the tin(II) and the concentration that would be expected in target organs after treatment.

The retention parameters (A_1 and A_2 in equation 1 and Figures 11 and 12) and their clearance half-times (T_1 and T_2 in equation 1) can be used to estimate the potential exposure that a mouse, or a particular tissue, can receive from a treatment. These values are important in calculating the integrated dose (equation 2) and are also useful in explaining cumulative concentration effects.

The metabolic fate describes how the chemical and physical form of the tin(II) behaves *in vivo*. In situ, tin(II) could be oxidized to inactive tin(IV), complexed to give some inactive form or excreted unchanged. The metabolic fate indicates how the cumulative concentration (C in equation 2) must be modified to

Figure II. The retention of ^{113}Tin(II) in mice.

Tin(II)-II3 was administered to mice by intraperitoneal injection. Retention of the radioactivity by the live mouse was determined at various times after administration by monitoring the whole body for gamma rays at 392 KeV. The shape of the whole body retention curve indicates that ^{113}Tin(II) is cleared in a biphasic manner. The initial retention for the long-lived component can be assessed directly by extrapolation of the clearance curve from infinite time to time zero.

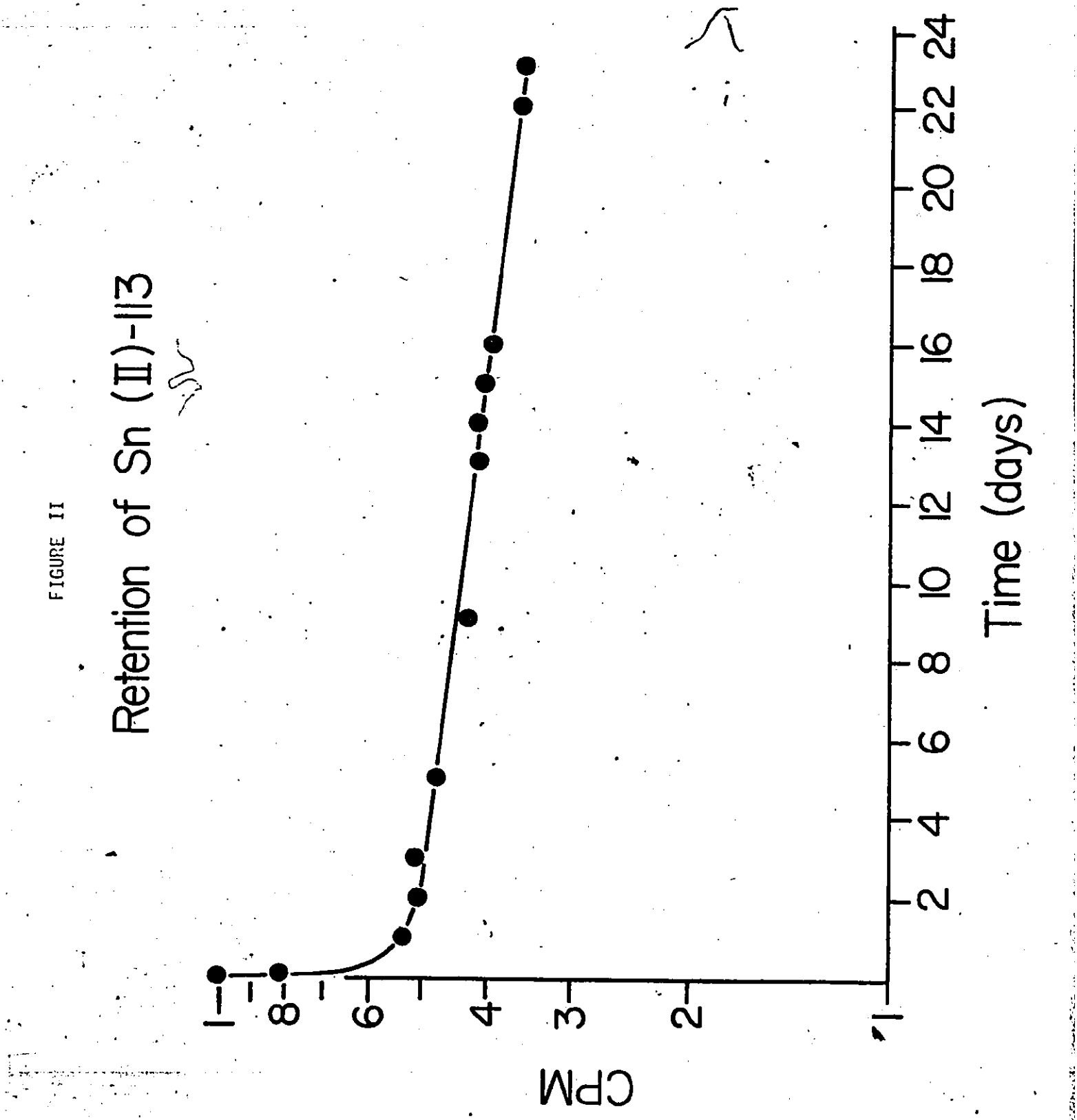

Retention of Sn (II)-113
CPM
8
6
4
3
2
1
2
4
6
8
10
12
14
16
18
20
22
24
Time (days)

FIGURE II

Fig. 12

Excretion of Injected ^{113}Tin (II) by Mice

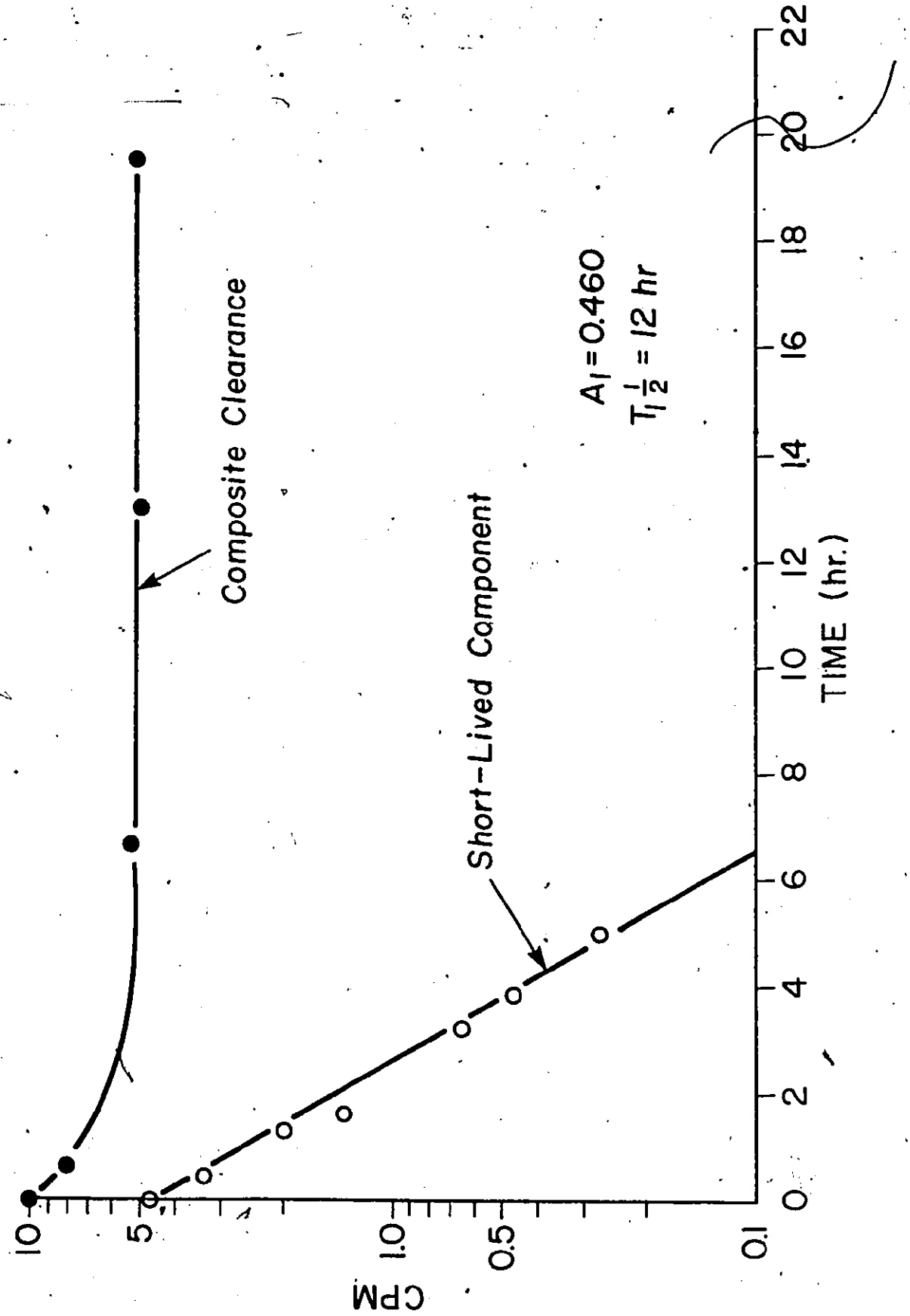


Figure 12. The excretion of injected ^{113}tin(II) from mice. The clearance (excretion) of radioactive tin(II) from mice after injection, can be described by a 2 component exponential equation (equation 1). The component that is cleared from the body with a short half-time can be resolved or "stripped" from the initial curvilinear portion of the composite curve (●) by subtracting the extrapolated values of the long-lived component. The resulting straight line (O) indicates that about 46% (retention parameter, A1 in equation 1) is cleared with a half-time of 12 hours. The balance of the injected radioactivity, 54%, would clear from the body with a half-time of 40 days.

Equation I

Clearance of ^{113}Tin (II) from mice

$$A(t) = A_1(0)e^{-\lambda_1 t} + A_2(0)e^{-\lambda_2 t}$$

where: $A(t)$ = RADIOACTIVITY AT TIME-t

$A_n(0)$ = RADIOACTIVITY AT TIME ZERO

λn = DECAY CONSTANT FOR COMPONENTS, n.1,2,...

e , LOG BASE e

Equation 2

Cumulative Tin (II) Concentration (μg-Days/Spleen)

$$\tilde{C} = C(0) Tb\tfrac{1}{2}\, e^{-\lambda t}$$

where: $\tilde{C}$ IS THE CUMULATIVE CONCENTRATION.

$C(0)$ IS THE INITIAL MAXIMUM CONCENTRATION

$Tb\frac{1}{2}$ IS THE BIOLOGICAL HALF-LIFE OF TIN(II).

λ IS THE DECAY CONSTANT

t IS THE TIME AFTER ADMINISTRATION OF THE DOSE.

reflect the inactivation or elimination of tin(II) from the body or organ.

4.5.1 The Clearance Half-Times and Retention Parameters of ^{113}Tin(II) in Black Mice

The clearance of ^{113}tin(II) from mice can be described as the sum of 2 exponentials, as in equation 1 (Cloutier 1976). A(t) represents the amount remaining at any time t, A_1 and A_2 are the retention parameters at time 0 and T_1 and T_2 are the biological half-times of the 2 components. Analysis of Figures 11 and 12, indicates that the rapidly clearing component has a retention parameter A_1 of 46% of the injected activity which clears from the body with a biological half-time of 12 hours. The slow clearing component has a retention parameter A_2 of 54% and clears from the mouse with a biological half-time of 40 days. Injection of nonradioactive tin(II) 8 days after the injection of ^{113}tin(II) did not increase the rate of excretion of radioactivity from the mouse indicating that the tin(II) of the slowly clearing component was firmly bound to cell structures.

4.5.2 The Tissue Distribution of ^{113}Tin(II) in Mice

The tissue distribution studies determine the important target tissues of tin(II) and also the maximum concentrations that can be found in these tissues. The tissue distribution of tin(II)-113 given intravenously at 0.5 mg kg^{-1} is shown in Table 25. The ^{113}tin(II) is rapidly cleared from the blood into the liver and spleen, with most of the dose being deposited in the liver. This tissue distribution pattern is characteristic of a colloidal preparation. Initially, there is little radioactivity in the bone and blood. The radioactivity is cleared only slowly from the liver and spleen.

The tissue distribution of ^{113}tin(II) given by intraperitoneal

TABLE 25

THE TISSUE DISTRIBUTION OF ^{113}TIN(II)
AFTER INTRAVENOUS INJECTION INTO MICE.

Tissue	TIME AFTER INJECTION (% of injected dose/organ-mean(range) 10 minutes	Day 1	3	5
Liver	92.3(0.1)	82.0(2.5)	86.0(3.5)	83.0(2.1)
Spleen	3.4(0.8)	3.0(0.9)	3.0(0.8)	2.6(1.0)
Lung	1.1(0.8)			
Kidney	0.4(0.0)	0.2(0.1)	0.2(0.1)	0.2(0.1)
Blood	0.4(0.1)			
Gut	0.3(0.1)			
Bone Marrow	1.0(1.0)			
Thymus	0.05(0.02)			

TABLE 25. THE TISSUE DISTRIBUTION OF ^{113}TIN(II) AFTER INTRAVENOUS INJECTION INTO MICE. A mixture of ^{113}Tin(II) and carrier tin(II), 10ug, was injected into the tail veins of mice. Mice were then sacrificed, in triplicate, at the times indicated, tissues were isolated and radioactivity measured in a gamma counter. Total blood volume was 6.9% of body weight. The distribution of radioactivity indicates that tin(II) is a colloid in solution1 and would concentrate to a large extent in the organs of the reticuloendothelial system. This indicates that after IV administration of tin(II) containing radiopharmaceuticals to humans, the target organ for tin(II) toxicity would be the phagocytic cells of the liver and to a much lesser extent, the spleen and bone marrow.

TABLE 26

THE TISSUE DISTRIBUTION OF ^{113}TIN(II)

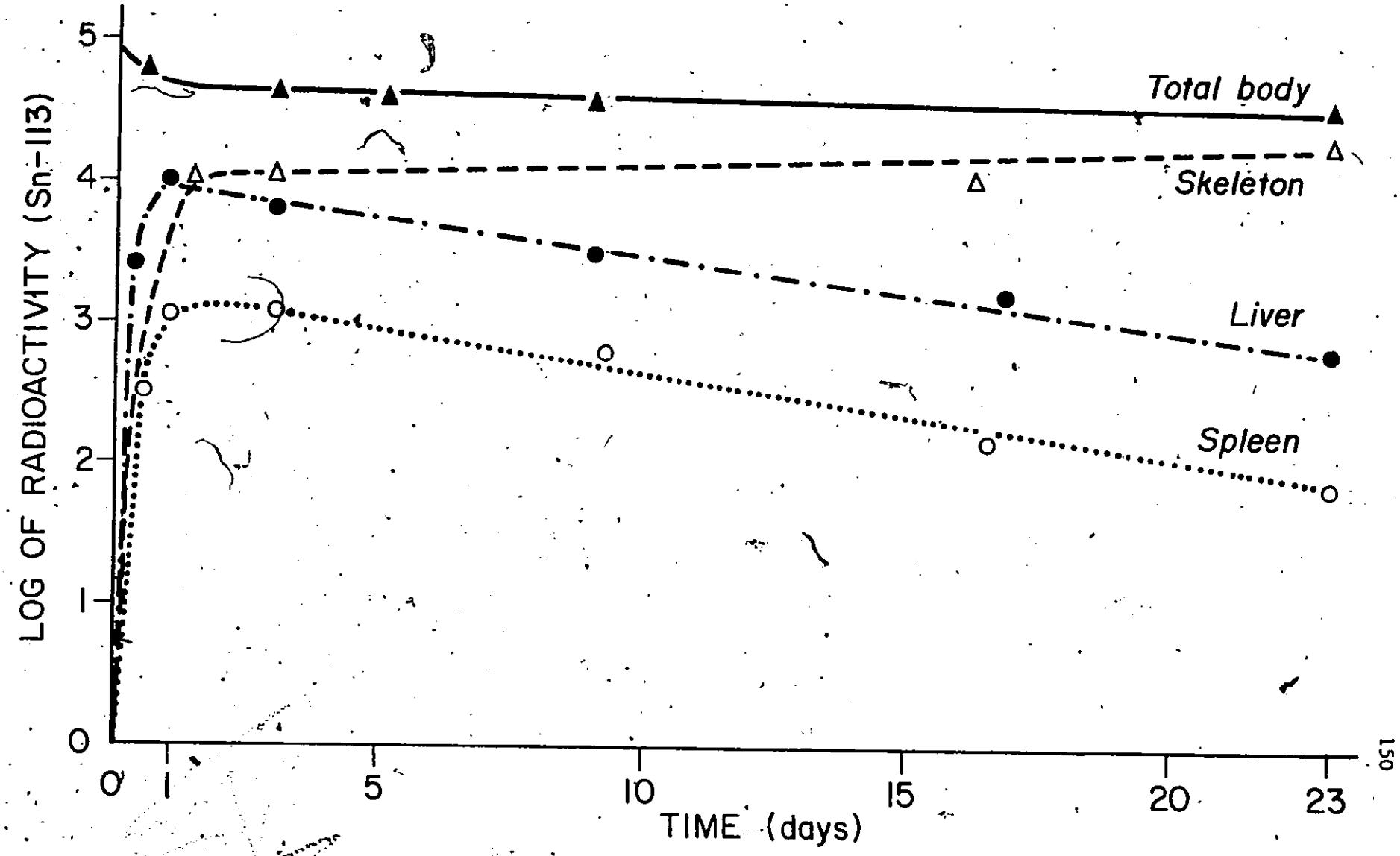
AFTER INTRAPERITONEAL INJECTION INTO MICE.

Tissue	Time After Injection (% of injected dose/organ-mean(range)			
	4 hours	Day 1	3	23
Liver	2.8(0.8)	12.2(7.8)	6.1(2.2)	0.7(0.1)
Spleen	0.3(0.1)	1.3(1.1)	1.1(0.8)	0.08(0.08)
Femur	-	0.1(0.1)	0.2(0.1)	1.2(0.2)
Kidney	-	0.8(0.8)	-	-
Blood	2.0(0.4)	0.6(0.1)	0.1(0.1)	-
Testes		1.4(1.0)	-	-
Gut		24.0(4.1)	-	-
Skeleton		8.1(1.1)	9.6(1.0)	38.5(3.3)

TABLE 26. THE TISSUE DISTRIBUTION OF ^{113}TIN(II) AFTER INTRAPERITONEAL INJECTION INTO MICE. A mixture of tin(II)-113 and carrier tin(II), 10ug, was administered to mice by intraperitoneal injection. Mice, in triplicate, were sacrificed at the times indicated, the organs isolated and the radioactivity in them was measured by gamma counter. Blood volume and skeletal mass were 6.9% and 6.5% of body weight respectively. The tissue distribution indicates that the liver and spleen accumulate tin(II), with the concentration in the liver being 6-12 times that in the spleen. The tissue distribution of tin(II)-113 after intraperitoneal injection is broadly comparable to that of tin detected in the organs of human cadavers, Kehoe et al.1940. This distribution, once again, emphasizes that the reticuloendothelial system is probably the target of tin(II) toxicity.

Fig. 13

Transport of ^{113}Tin (II) Radioactivity from Liver and Spleen to Skeleton in Mice



150

Figure 13. The transfer of injected ^{113}tin(II) from soft tissue to skeleton of mice. Initially, the uptake of radioactivity into the skeleton occurs quickly but after a plateau is reached increases occur only slowly with time as radioactivity is lost from the soft tissues (liver and spleen). The rate of this loss is 4.03% per day of the radioactivity retained in the organ.

injection at 0.5 mg kg^{-1} is shown in Table 26. The distribution of ^{113}tin(II) is relatively uniform throughout the body and once again, the liver and spleen retain their activity for prolonged periods. ^{113}Tin(II) is slowly lost from the liver and spleen at 4.03% of the retained organ activity/day, Figure 13. ^{113}Tin(II) accumulates in bone with time, probably as it is lost from the soft tissues of the body. If tin(II) retained its reactivity (electronic configuration or valence) *in vivo* then the time component in the dose equation would be an important determinant of the cumulative dose.

4.5.3 The Metabolic Fate of Tin(II) In Vivo

The metabolic activity of tin(II) *in vivo* is important in determining how the time factor in the dose equation should be modified. Tin(II) is a powerful reducing agent and this property can be used to obtain an estimate of how reactive tin(II) remains *in vivo*. ^{99m}Technetium, Tc(VII), is a gamma emitting radionuclide that remains chemically unreactive until it is reduced by tin(II) to highly reactive ^{99m}Tc (III) or (IV). Therefore, when ^{99m}Tc(VII) is injected into tin-free mice, it is distributed uniformly throughout the body and is then rapidly excreted, primarily by the kidneys. If tin(II) has been previously administered to the mouse, the distribution and clearance pattern of ^{99m}Tc is dramatically changed by the reducing power of tin(II). The loss of this modified distribution pattern with increasing time after the injection of tin(II), indicates how the reactivity of the tissue-bound tin(II) is changed by loss through intracellular oxidation to tin(IV), by complexation or by excretion.

The difference in ^{99m}Tc radioactivity in the organs of control mice (no tin) and mice treated with a single intraperitoneal injection of tin(II) 96 hours before the administration of ^{99m}Tc(VII), is shown in Table 27. The differences in radioactive uptake of ^{99m}Tc in various tissues for the 2 groups were tested at

the 98% level of significance by the method of Wang, 1969. The differences are statistically significant if;

(A counts) - (B counts) $\geq \sqrt{2(A+B)}$, where A and B are radioactive counts from the 2 organs (±tin) being compared. Using this criterion, the distribution of ^{99m}Tc in the blood, liver, kidney, spleen, muscle, mesentary, testis and stomach of tin(II) treated mice were statistically different from controls, while the distribution in the skeleton, femur, bone marrow, small intestine, large intestine, seminal vesicles and lungs were not. The tin(II) treatment suppressed the uptake of ^{99m}Tc by the stomach.

The in vivo reactivity of tin(II) given to mice for 9 months in their drinking water at 100 ppm was monitored in a similar way. Reference to Table 28 shows that tin(II) is absorbed after oral administration and distributed systemically. Although the uptake is small, significant accumulation did occur in bones, bone marrow and spleen.

The distribution patterns of $^{99m}Tc(VII)$, in the presence or absence of tin(II) is mainly a qualitative test but it does reinforce the idea that tin(II), given orally or by injection, remains reactive in the body. This suggests that the "time" component of the dose equation makes a significant contribution in the calculation of dose.

TABLE 27

THE DISTRIBUTION OF ^{99m}Tc-PERTECNETATE IN MICE 96 HOURS AFTER THE ADMINISTRATION OF TIN(II) AT 7 MGKG^{-1} BY INTRAPERITONEAL INJECTION.

Organ	% Of Injected Dose /Organ(mean standard deviation) Control Mice	Tin(II) Treated Mice	Tin(II)/Control.
Blood	4.20 ±1.10	17.20 ±2.50	4.1
Liver	3.30 0.10	20.20 3.00	6.1
Kidney	0.60 0.10	2.20 0.10	3.7
Spleen	0.07 0.02	0.45 0.20	6.9
Muscle	2.10 0.10	5.40 3.50	2.6
Skeleton	2.10 0.70	3.20 2.50	1.5
Femur;			
ends	0.04 0.02	0.07 0.05	1.4
marrow	0.01 0.00	0.01 0.00	0.8
Intestine;			
small	13.40 0.90	9.40 0.50	0.7
large	22.40 6.70	12.70 1.30	0.6
Stomach	12.10 4.20	2.00 0.20	0.2
Mesentary	0.25 0.05	1.25 0.05	5.0
Testes	0.09 0.00	0.30 0.30	3.3
Seminal vesicles	0.65 0.20	1.10 0.60	1.7
Lungs	0.45 0.10	1.10 0.30	2.4

TABLE 27. THE DISTRIBUTION OF ^{99m}Tc-PERTECHNETATE IN MICE 96 HOURS AFTER THE ADMINISTRATION OF TIN(II). Mice were injected with carrier-free ^{99m}Tc(VII), sacrificed 2 to 4 hours later and the organs were isolated and assessed for radioactivity. The ^{99m}Tc(VII) is reduced by the tin(II) and is then fixed to tissue components. The concentration of Tc injected is many orders of magnitude lower than that of the tin(II) in the tissues. Therefore, the distribution of the Tc-99m in these animals largely reflects the volume and rate of blood flow in the tissues. The study, indicates that the reducing power of tin(II)(ie the valence) remains intact in the tissues and is not readily oxidized or complexed to inactive forms. This suggests that the time component of the dose equation contributes substantially to the calculation of the total (integrated) dose from the presence of tin(II) in tissues.

TABLE 28

THE EFFECT OF ORALLY ADMINISTERED TIN(II)

ON THE TISSUE DISTRIBUTION OF 99mTC(VII).

% of Injected Dose/Organ(mean)

Organ	Control Mice	Tin(II)Treated Mice	Tin(II)/Control
Blood	0.2000	0.4000	2.0
Liver	0.8000	1.0000	1.3
Kidney	0.0800	0.3000	3.8
Spleen	0.0040	0.0300	7.5
Muscle	0.1300	0.5000	3.9
Skeleton	0.1200	12.2000	102.0
Femur;			
ends	0.0020	0.2000	100.0
marrow	0.0004	0.0500	125.0
Intestines;			
small	0.9000	0.0600	0.7
large	3.0000	2.3000	0.8
Stomach	1.3000	1.4000	1.1
Mesentary	0.0200	0.0700	3.5
Testes	0.0100	0.0500	5.0
Seminal vesicles	0.2000	0.0900	2.2

TABLE 28. THE EFFECT OF ORALLY ADMINISTERED TIN(II) ON THE TISSUE DISTRIBUTION OF Tc99m(VII) PERTECHNETATE. Mice were administered tin(II) in drinking water at 100 ppm. Mice were given intraperitoneal injections of carrier-free Tc99m(VII) 24 hours prior to sacrifice. The pretreatment of mice with oral tin(II) changed the distribution pattern of the 99mTc and indicates that considerable reducing power can be retained in the spleen, kidneys, muscle, skeleton, marrow and testes. This study shows that tin(II) remains active in-situ for prolonged periods, and suggests that some tin from the environment could accumulate at sensitive sites in the body after being converted to tin(II) by the action of the gastric HCl.

4.5.4. The Detection of DNA Damage In Vivo by the FADU Technique.

A relevant extension of the *in vitro* experiments is to see if DNA damage can be detected in the organs of mice that have been treated with tin(II). One shortcoming in the use of an *in vivo* system involves the presence of a large number of uncontrolled and interacting variables that can give results that are often ambiguous and difficult to interpret. Nevertheless, rigorous toxicity testing requires the use of one or more mammalian species in some aspects of the evaluation program.

The DNA damage produced in cells by Co-60 gamma rays was used as the reference for comparing the damage produced in the DNA of cells treated with tin(II). The spleen was used as the target organ because it accumulates tin(II), Hiles 1974, and Hamilton et al, 1972/73, and it is easy to process for FADU analysis. DNA damage from whole body exposure to cobalt-60 gamma has been detected in spleen, bone marrow and thymus, McLean et al, 1981. The response, as measured by FADU is linear with respect to doses from 1 to 3 Gy.

DNA damage can be detected in the spleens of mice that have been injected intraperitoneally with tin(II) at 7mg/kg, figure 14. The DNA damage is in the range that is produced by exposure of the mouse to 2 Gy of Cobalt-60 gamma rays. The uptake of tin(II) by spleen is estimated to be about 1% of the injected dose, which for a 20 g mouse would be about 1.5 ug/spleen. This would be about 0.03 fM/cell, assuming there is 400 $x10^6$ cells/spleen. The study by Lawrence, 1981 found a statistically significant inhibition of the immune response *in vitro* by treatment with tin(II) at less than 0.1uM. The binding of tin(II)per cell at

this concentration, as estimated from my experiments would be about 0.04 fM. A part of the study by Lawrence involving tin(II), is severly compromised because of a procedural error and the actual concentration of tin(II) in the incubation medium may be several times lower than reported. It appears that tin(II) is very potent, biologically, to some tissues at low doses.

The tests on the mice described here have to be considered only as preliminary. The mechanism by which tin(II) exerts its biological effects in vivo is not known but 2 alternative hypotheses can be proposed. In one, tin(II) would produce direct DNA damage which could explain the observed effects on the immune system in vivo.

In the alternative hypothesis, tin(II) could be cytotoxic under the conditions of high cell density found in vivo and the observed effects on the immune system could be explained on the basis of a selective loss of cells undergoing DNA synthesis. The strand breaks, in this case, could be from DNA disassembly in a dead or dying subpopulation of cells. This is an obvious area where extended studies could be undertaken.

Fig. 14

DNA Damage to Mouse Splenocytes <u>in vivo</u> After Exposure to Tin (II), 7 mg Kg^{-1} (○) or 2 Gy of ^{60}Co-Gamma Rays <u>in vitro</u> at 0°C (△) and <u>in vivo</u> (●)

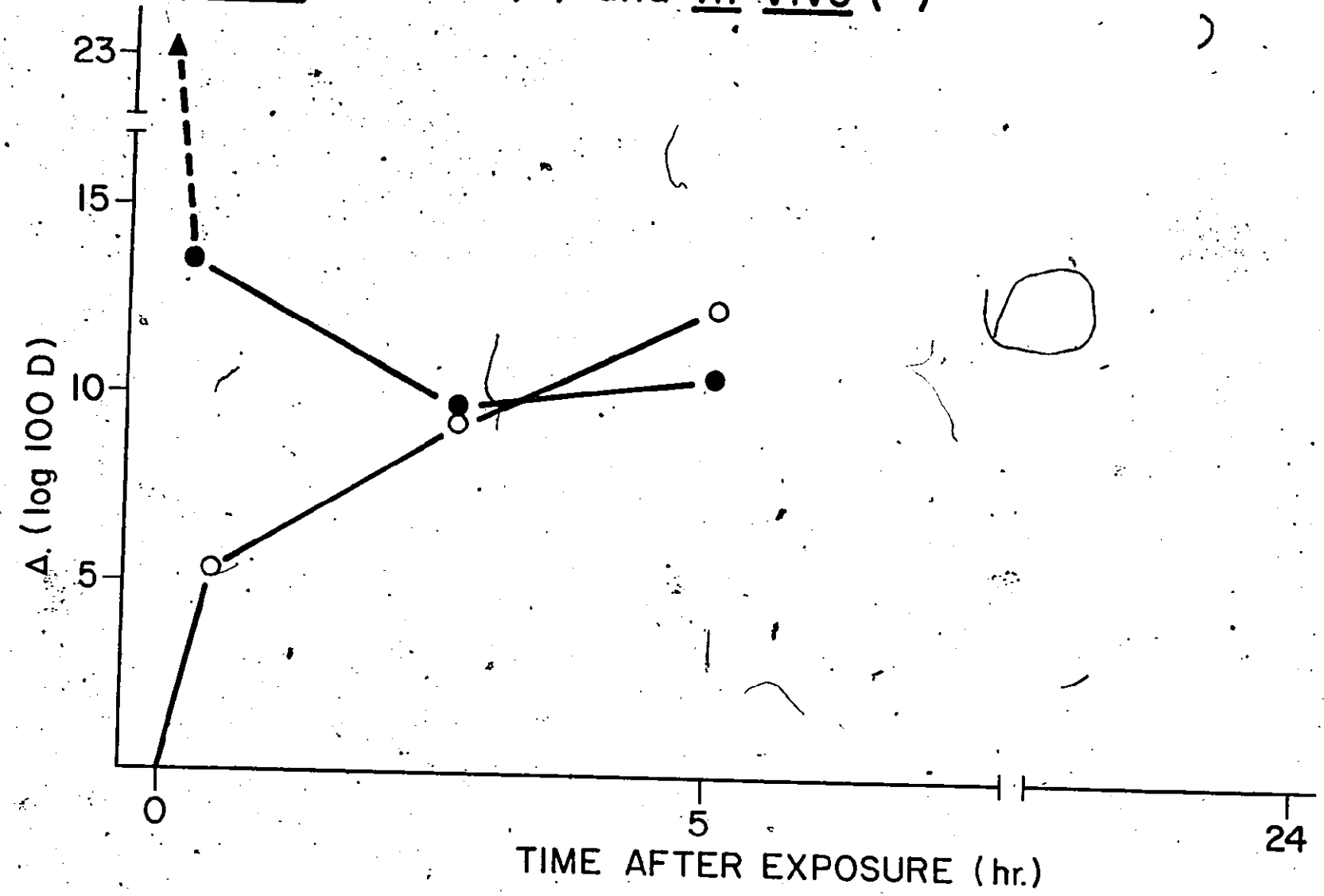


Figure 14. DNA damage in mouse splenocytes exposed to tin(II) in vivo. DNA damage is illustrated in mouse splenocytes exposed to tin(II) in vivo ( ) and to 2 Gy of cobalt-60 gamma rays in vitro ( ) and in vivo ( ). Damage was detected in splenocytes one hour after the injection of tin(II) and increased steadily with time. Repair of the damage was not detected. The observed damage could represent frank breaks in the DNA chain which would increase as tin(II) accumulated in the organ. Alternately, the observed breaks could be due to DNA disassembly occurring as a result of cell death, Williams 1976.

5.0 DISCUSSION AND CONCLUSIONS.

5.1 THE UPTAKE OF TIN(II) BY CELLS.

The up take of ^{113}tin(II) by cells suspended in BSS occurs in 2 phases; a rapid saturable phase (K_m=18 uM, Vmax=417 fg/cell/min) which predominates at high tin(II) concentrations, >10uM, and a slow phase of more prolonged uptake which does not appear to be saturable and which predominates at low Sn(II) concentrations. Only the latter phase would be relevant at the low Sn(II) concentrations that occur under environmental and clinical conditions. This dual uptake process is characteristic of many substances, including anions.

The uptake of Sn(II) by cells suspended in BSS, is largely by passive diffusion. This is supported by the Q_{10} value of 1.03 and by the fact that Sn(II) and many of its chelates are rapidly taken up by cells at 0°C. Once inside the cell, Sn(II) could bind to a variety of intracellular components, creating a persistent diffusion gradient that would insure a steady influx of Sn(II). Small particles of Sn(II) can also be quickly taken up by the cells creating depots which can be a constant source of intracellular Sn(II).

Throughout this study, it has been consistently found that the uptake of Sn(II) *in vitro* and all of the related responses or effects, cannot be related to the external Sn(II) concentration by a simple linear function. There is a "cooperative-like" effect where uptake and the resulting responses become exaggerated at concentrations >10uM tin(II). This effect may be due to a chemical or physical effect localized at the membrane. Many tin(II) chelates, in aqueous solutions, readily form molecular and colloidal sized aggregates at pH 7 (Nelp,

1969). The size distribution of the aggregates depends on the concentration of Sn(II) in the extracellular medium. The presence of these particles, near or on the membrane surface could create localized areas of Sn(II) acitivity. These localized areas which could perturb the physical integrity of the lipid bilayer or the functional capacity of membrane bound enzymes, may be the physical or chemical channels that allow the rapid influx of small particles and molecular aggregates. The size of the perturbed area in the membrane would vary as a function of the volume ($radius^3$) or surface area ($radius^2$) of the particle. The result of these localized membrane perturbations would be a "cooperative-like" effect in which progressively higher concentrations of tin would produce proportionately larger localized perturbations resulting in even greater influxes of the smaller aggregates of Sn(II). Such an uptake has been observed for CdO particles (Hadley, 1980) and curium dioxide particles (Stradling, 1979 and Cooper, 1980).

Alternately, the cooperative-like effect may be nothing more than an artifact of the chemical reactivity of Sn(II) in dilute solutions. At high Sn(II) concentrations, saturation of the numerous external non-specific binding sites (glass and plastic surfaces and tissue fragments) would occur with little loss in the Sn(II) available for cell uptake. These external binding sites directly compete with cellular sites for Sn(II) and would become limiting as the Sn(II) concentration in the extracellular medium progressively decreased.

The uptake of Sn(II) by the cell can be modified by the presence of a variety of compounds that can form coordination complexes with the Sn(II). The rate at which the Sn(II) complex penetrates the cell membrane is largely determined by the lipophilic and charge characteristics of the ligand.

Tin(II)-EDTA does not penetrate the cell whereas, complexes of F_2, phosphate, citrate, phytate, Cl_2, CDTA, MDP and PyP readily penetrate the cell membrane at both 0^oC and 37^oC (MDP penetrates at 37^oC only). Sn(II) phytate and Sn(II) F_2 form colloids at pH7 with particle sizes ranging up to 10 nm. (Personal communication, Amersham, Ltd). The fact that they are rapidly taken up by cells argues against uptake by pinocytosis or phagocytosis. Tin(II) binds strongly to HSA and the complex can be precipitated with 5% TCA. The uptake of Sn(II) in the presence of protein is slow suggesting that uptake is mediated by pinocytosis. The Sn-HSA complex would be taken up into lysosomes and subsequent digestion of the protein would release Sn(II) into the intracellular space. In some experiments, damage from Sn(II) in the presence of 6% FCS was detectable in cells after 12 hours of incubation at 37^oC.

5.2 THE EFFECT OF TIN(II) UPTAKE ON DNA DAMAGE IN VITRO.

The observations that Sn(II), directly damages DNA in cell lysates, in cells at 0^o and 37^oC, and damages cellular DNA in the presence of 2-DOG, indicates that the action of Sn(II) against DNA is direct and is not a manefestation of enzyme controlled DNA disassembly or cytotoxicity mediated through neutrophil excretion of H_2O_2 in response to the presence of particles.

The ability of Sn(II) complexes to damage DNA depends on the extent to which the complex can penetrate the cell with at least 2 reactive orbitals of the Sn(II) occupied by weakly bonded groups, such as chlorine. The Sn(II) complexes of phosphate, citrate, Cl^-, CDTA, F_2 and phytate can penetrate the cell at 0^oC and 37^oC and can react with DNA to cause extensive damage. Tin(II) MDP and CDTA are very efficient at producing DNA damage, relative to Sn alone, being able to

produce 57,706 and 3,517 breaks/fM in relation to 979 for tin(II) complexed to Cl^- The complex of Sn(II)-Pyrophosphate$_2$ penetrates the cell but does not cause DNA damage because all bonding orbitals of Sn(II) would be occupied by a ligand with a large binding constant.

The DNA damage observed at high Sn(II) concentrations, however is not linearly related to the uptake process because Sn(II), when present in the cell, actively suppresses the repair of strand breaks. The, evidence for a graded inhibition of repair by increasing Sn(II) uptake is derived data from table 13.

The difference in breaks/genome at $0^{o}C$ and $37^{o}C$ is shown in tables 12 and 13. The observed differences could be due to:

1) the breaks caused from the increased Sn(II) uptake at $0^{o}C$ (if it is real), and

2) strand break repair which would occur simultaneously with the generation of strand breaks at $37^{o}C$ but not at $0^{o}C$ where rejoining is inhibited.

The increase in uptake of Sn(II) at $0^{o}C$ over that at $37^{o}C$ is 0.76 and 0.04 fM/cell from solutions containing 50 and 25 uM Sn(II) respectively. The number of strand breaks generated from this uptake can be calculated by the formula:

strand breaks = Breaks/genome/fM x fM/cell

Taking the data from tables 12 and 13, this comes to 959 and 35 strand breaks for 0.76 and 0.04 fM/cell respectively. This leaves 1634 and 274 respectively (2593-959 and 309-35) that cannot be explained on the basis of differences in Sn(II) uptake at the 2 temperatures. The differences however can be explained on the basis of strand break repair which would be occurring at $37^{o}C$ but not at $0^{o}C$. The differences between expected values 959 and 35 and the observed values 2593 and 309 can be formulated into a null hypothesis and tested by the Chi statistic. The difference by X^2 at p= 0.001 is highly significant and an altenative hypothesis, that the observed differences are due to repair should be accepted at least as a plausible area for further exploration. Further evidence that tin inhibits strand break repair comes from the generation of breaks/fM, table 13.

The number of breaks generated /fM decreases rapidly as the Sn(II) concentration decreases indicating that there is a greater rate of strand rejoining at low Sn(II) concentrations. From table 12 all of the strand breaks are repaired within 20 minutes after Sn(II) at 5 - 50uM is thoroughly washed from the cells. This means that the 7458 breaks observed in the presence of Sn(II) at 50uM will be repaired within 20 minutes at an average rejoining rate of 371 breaks/min. This is considerably faster than 86 breaks/min. occurring during the 30 minute treatment period with the Sn(II) present. The actual break and rejoining would be a dynamic on-going process with a constant number of breaks being generated per minute once the maximum level of Sn(II) had been reached; the rate of rejoining would concomitantly drop to some constant inhibited value. The rates of these processes would slowly reverse as Sn(II) diffused out of the cell or was oxidized to inactive Sn(IV) or was complexed to some inactive form. The Sn(II) level at which the rate of breakage equalled the rate of strand rejoining would be the Sn(II) concentration for which damage would not be detectable at 37°C. At low levels of Sn(II), uptake into the cell would be small and slow enough that the repair process would not be saturated and strand breaks could be generated without being detected. Some estimate of the rate of repair can be obtained by studying damage which is not fully repaired. In table 18, the sequential insult of DNA by Sn(II) at 25uM and 2 Gy Co-60 produced and additive damage of Δ (100 log D) = 121 or 8989 strand breaks/genome. The residual damage remaining after a 20 minutes incubation at 37°C was Δ (100 log D) = 17.6 or 1307 residual strand breaks per genome. The rate of repair therefore was in the order of 384 breaks/min which is close to the rates discussed previously.

The FADU and ASG use an alkaline denaturation step and so primary strand break damage cannot be distinguished from secondary breaks induced in DNA by

repair endonucleases or by alkaline cleavage at sites of base damage. Therefore, Sn(II) could produce a variety of damages in DNA. Tin(II), like Fe(II)/Fe(III) and Cu(I)/Cu(II), could generate H_2O_2 or the superoxide anion in the incubation medium or catalyze the conversion of H_2O_2 to the more damaging hydroxyl radical. Tin(II) can combine with Fe(III) or Cu(II) using its $5s^2$ electrons to form a regenerating redox couple of the type Sn(III)/Fe(II) (Binder, 1979). Tin(II) dichloride has a configuration very close to that of cis-platinum. The weakly bonded chlorines can be readily displaced by more strongly bonding O_6 and N_7 positions of guanosine and the pyrophosphate backbone and the deoxyribose sugars likely sites for Sn(II) bonding. This type of bonding, which would be partly covalent in character would produce a distortion or bulky lesion in the DNA that would also be capable of inhibiting DNA synthesis.

5:3 THE EFFECT OF TIN(II) ON DNA SYNTHESIS.

The inhibition of DNA synthesis by Sn(II) is rapid in onset and prolonged. The repair of strand breaks is inhibited by the presence of high concentrations of Sn(II) but occurs readily when the concentration of extracellular Sn(II) is reduced by rigorous washing procedures or as it is converted to the inactive Sn(IV) by intracellular oxidation. Persistent lesions, capable of blocking DNA synthesis by inhibiting chain initiation and chain elongation, could arise in several ways. First, bulky lesions arising from the covalent binding of Sn(II) to the bases and backbone of DNA could theoretically occur. Second, unrepaired strand breaks could occur due to continuous presence of active intracellular Sn(II).

Blastogenesis in vitro, is inhibited 50% by treatment of resting HPBL and

mouse splenocytes with 20 and 30 uM Sn(II). This represents binding of about 3.0 fM/cell for about 0.5 - 1.0 hours before concanavilin A is added to the cultures. This would introduce about 3210 strand breads/genome into the cells. According to Painter 1980, about 3600 random breaks per genome is sufficient to completely inhibit DNA synthesis in mammalian cells. The presence of intracellular Sn(II) particles (of colloidal or molecular size) would allow these breaks to persist by inhibiting repair and by supplying fresh Sn(II) to generate new breaks. In addition, Butzow 1975, has shown that single stranded DNA and RNA are many times more susceptible to cleavage by metal ions than is double stranded DNA. Wong et al., 1974 has shown that the Cu(I)/Cu(II) couple rapidly cleaves phage RNA in situ, probably through the intermediary of the hydroxyl radical. Therefore, Sn(II) may have the capability of converting the persistent single strand breaks to double strand breaks and cleaving RNA; both effects would have long-term implications for the repair and synthesis of DNA.

About 1 uM Sn(II) is sufficient to inhibit DNA synthesis by 50% in mouse EL-4 cells. These transformed cells appear to be 20 fold more sensitive to Sn(II) than resting cells. This observed effect, like the high efficiency of strand breaking produced by Sn(II) MDP and CDTA, are areas of potential practical interest that will be explored in the future.

5.4 THE EFFECT OF TIN(II) IN VIVO.

The observed effects of Sn(II) on cells in vitro, occur at relatively high concentrations and under conditions of cell density which would never be found at environmental or clinical exposures. In order to show relevance, it is necessary to demonstrate that these effects occur in vivo under conditions where the

exposure to Sn(II) is much lower. Four effects have been demonstrated in this thesis after in vivo exposure of mice to Sn(II). Three of these effects are related to the suppression of immune function and the fourth involves detection of DNA damage in spleens of Sn(II) treated mice. The objective of this study was to show that the inhibition of immunological function can be explained on the basis of the fourth effect, that of DNA damage to those cells that are essential for the development and functioning of immune system.

Tin(II), administered by intravenous or by intraperitoneal injection, is retained in the tissues of the body for a prolonged period of time, and only slowly eliminated via the urine and feces (Furchner, 1976) and in figures 25 and 26. The Sn(II) that is taken up by the oral route (approximately 1-3% of administered dose) is also retained in the body. After I.V. administration, Sn(II) accumulates 90% of the injection in the RES (liver and spleen). The initial tissue distribution after I.P. administration is more uniform, being evenly distributed throughout the body, with a noticeable accumulation in some tissues of the RES. The distribution of Sn(II) by the I.P. route, is similar to, but an exaggeration of, the distribution obtained from a single oral dose (Hiles, 1974 and Furchner, 1976) and therefore may be used as a first approximation of the distribution that might be obtained from chronic oral exposure to Sn(II). Initially, little Sn(II) is found in the skeleton, but with time, it is transferred at a rate estimated to be about 4% of the total organ burden per day. The Sn(II) that accumulates in the bone remains active with a half-life of more than 13 weeks (McRae, 1976) and figures 11 and 12. Tin(II) that is taken up in organs after oral administration retains its reactivity and valency. This can be demonstrated by the change in the tissue distribution pattern of $^{99m}TcO_4^-$ in mice that have been treated with oral Sn(II).

The excretion of tin in the urine, represents intracellular Sn(II) that has been oxidized to Sn(IV) and that in the bile, Sn(II) that has been bound to heme degradation products. The overall excretion rate, after the initial 12 hour rapid phase, is 1% of the total body burden per day or 0.5% of the injected dose. The observation that Sn(II) remains reactive in the body for a prolonged period was demonstrated by McRae, 1976 and confirmed and extended in the tables 27 and 28. The assumption that Sn(II) remains active in the body and is only slowly metabolized to inactive Sn(IV) or Sn-heme complexes, permits the use of the integrated (or cumulative dose concept) (Cloutier, 1976) to explain the observations of DNA synthesis inhibition and DNA damage at the low Sn(II) concentrations found *in vivo*. In using this concept, several assumptions must be stated:

1) the rate of elimination of Sn(II) from the mouse spleen is 4.03% of the organ burden per day, (figure13, that is, it has a biological half-life in the spleen of 17.1 days.

2) the rate of conversion of Sn(II) to inactive Sn(IV) in the spleen is low because the overall elimination of tin (presumably Sn(IV)) in the urine is small.

3) uptake of Sn(II) in the spleen is instantaneous;

4) the rapid elimination phase, which occurs during the first 24 hours after injection is not included in the calculations.

5) the uptake of Sn(II) into the spleen is 1.4% of the intraperitoneal dose, table 26;

6) the spleen of a 20g mouse contains 400 x 10^6 nucleated cells;

7) the injection of Sn(II) does not cause any physiological changes, such as cell renewal, granulopoesis etc.

The cumulative Sn(II) concentation in ug-day/spleen is given by equation 2.

Briefly, the cumulative concentration can be described as the initial concentration in the target tissue x time, with an adjustment for biological elimination or inactiviation of Sn(II) by metabolism to Sn(IV). The integrated dose however must be adjusted to reflect treatment times. In vitro assays for strand breaks were done at 0^oC to determine the total number produced after 0.5 hours of incubation. About 1,262 breaks/fM/0.5 hours are produced, table 13 and this must be multiplied by 48 to get the total damage, in terms of strand breaks, for a 24 hour period.

Table 29

Intregrated concentration of tin(II) at end of D_n days.

(ug-days/whole spleen)

Dose mg/kg.	C(0) T_b0.5	Initial Uptake	D0	D1	D2	D3	D5	D7
1.0	5.13	0.3	.21	.41	.67	.77	.92	1.23
3.0	13.68	0.8	.56	1.1	1.8	2.1	2.5	3.3
5.0	23.94	1.4	1.0	1.9	3.1	3.6	4.3	5.8
7.0	34.20	2.0	1.4	2.7	4.4	5.1	6.2	8.

Integrated dose calculated according to equation 2.

In the immunological in vivo experiments, Sn(II) was injected 2 days before SRBC and assays for DNA synthesis and PFC's were done at day 7. The cumulative doses are shown in table 30.

TABLE 30

INTEGRATED DOSE EXPRESSED AS TOTAL DAMAGE (STRAND BREAK-DAYS/GENOME)

Dose,mg/kg.	Integrated Dose,fg/cell		Total Breaks/Genome Time(D) After Tin	
	Day-2	Day-7	Day-2	Day-7
1.0	1.7	3.1	824	10,520
3.0	4.4	8.2	2,618	27,827
5.0	7.8	14.4	7,563	48,868
7.0	11.1	20.5	10,763	69,569

According to Painter, 1975, one strand break per 10^9 daltons is sufficient to prevent replicon initiation (3600 breaks/genome) by 100%. Therefore, the total damage induced, disregarding repair, can explain the suppression of DNA synthesis. The strand break damage could represent frank single strand breaks, base damage and double strand breaks. The greater the number of single strand breaks produced the greater the probability that double strand breaks will occur. Double strand breaks are much slower to repair and they can produce a prolonged inhibition in DNA synthesis, Leenhouts, 1978.

The DNA damage observed after Sn(II) injection may be due to strand breaks, like the in vitro situation or it could represent cell death and DNA disassemble. The spleen cells of rodents are extremely sensitive to low doses of radiation (Geraci, 1975) (Jackson et al.,1969). This problem remains to be resolved, but the effect, whatever its nature is prolonged, figure 14.

5.5 THE DOSE AND RISK ESTIMATES IN HUMANS FROM ENVIRONMENTAL EXPOSURE TO TIN(II).

There is little factual information on the concentration of Sn(II) in humans. Data compiled by Schroeder et al., 1964, is based on a 1940 analysis (Kehoe, 1940) of tin in the organs of cadavers. No distinction was made between Sn(o), Sn(II) or Sn(IV), indeed it has been only recently that a sensitive and reliable assay for Sn(II) has been developed, Silva et al., 1980. It is interesting to note that tin was taken up very rapidly in infants. Analysis by Hodge et al., 1979 on tin in drill cores taken from marine sediments in the U.S.A. indicate that tin rapidly accumulated in the environment after the 1940's correlating with its expanded use in the production of plastics, toothpastes, radiopharmaceuticals and as a preservative in foods and beverages. Drill core samples estimate that tin increased in the environment at an annual rate of 1 ppm between 1962-1972, reaching 20 ppm. There are no estimates beyond this date. The concentration of tin in cadavers analyzed in 1940 occurred largely when there was little tin pollution (10 ppm). In 1940, tin was found in all organs surveyed. The liver accumulation was highly variable but a figure of 25 ppm (ash) appears to be representative. This concentration will be used as the base for estimating current doses in the human spleen, In mice, the amount of Sn(II) accumulated per gram of tissue is about equal for spleen and liver . The 25 ppm spleen would have accumulated as a result of exposure to environmental tin (used

in its widest meaning) at about 1-13 ppm during the pre-1900's to late 1930's. This exposure more than doubled by 1972 and using the best estimates from 1967-1972, the increase can be projected at about 1 ppm/year until 1981 to give about 30 ppm. If 13 ppm (maximum estimate) by 1940 gave a spleen accumulation of 25 ppm, then the increased environmental exposure would give a current spleen burden of 58 ppm (ash) or (using the Schroeder conversion factor, 1.64/150) about 0.63 ppm wet weight. If one gram of human spleen contained 10^{10} cells, then the concentration would be 0.063 fg/cell. Data from in vitro studies indicate that 1fg tin(II) introduced 10.1 strand breaks in 0.5 hr then 0.063 fg/cell would generate 10995 strand break-year/cell, assuming equilibrium between uptake and inactivation. An exposure of 0.01 rads/day (0.0001 Gy/day) whole body is 5 R/year, the upper limit for occupational exposure to radiation workers, gives 0.01 rads/day x 1196 x 360 = 4306 break-year/cell. The exposure to the spleen then is equivalent to about 12.8 rads/year (0.128 Gy/year). Chronic exposure from natural background radiation is about 0.001 rads/day or abour 431 break-year/cell. (Biological Databook, 1965). The assumption that all tin in the cell is Sn(II) is an obvious overestimate but is not unrealistic since only Sn(II) appears to readily get into the cell and Sn(II) is much more readily absorbed orally than is Sn(IV), Hiles, 1974. There is also evidence showing that tin metal, Sn(0) is slowly converted to Sn(II) in acidic solutions, (Bailer, 1974) such as the gastric HCl. Nothing is known about the rate at which Sn(II) is inactivated to Sn(IV) in the cell or whether Sn(II), sequestered in the skeleton, can be redistributed to the rest of the body under pressure of hormonal changes.

5.6 Future Directions

Preliminary results that have been obtained, but not reported include the following:

1) a small but statistically significant rise in sister chromatid exchange rates in CHO cells treated in vitro with Sn(II) concentrations less than 10uM.

2) observation of spermhead anomalies in mice treated in vivo with Sn(II) in the range of 5-7 mg kg^{-1}.

These observations, the apparent *in vivo* DNA damage, and the effect of Sn(II) on RNA will be investigated in the future.

REFERENCES

Abel, E. W. (1973), In: "Comprehensive Inorganic Chemistry,: V. 2, Pergamon Press, Eds. Bailar, Jr., J. C., Emeleus, H. J., Nyholm, Sir R. and Trotman-Dickenson, A. F., pp. 43 - 104.

Abel, E. W. (1978). "Structural tin chemistry." Prog. in Inorganic chemistry, 24: 271 - 451.

Adams, R. L. and Lindsay, J. G. (1967). "Hydroxyurea: reversal of inhibition and use as a cell synchronizing agent". J. B. C. 242: 1314 - 1317.

Alexander, P. and Horning, E. (1958). "Observations on the Oppenheimer method of inducing tumors by subcutaneous implantation of plastic films." In: Wolstenhikme, G., O'Connor, M., eds. Carcinogenesis, mechanism of Action. Boston: Little, Brown, (1958): pp. 12 - 22.

Ames, N., Lee, F. and Durston, W. (1973). "An improved bacterial test system for the detection and classification of mutagens and carcinogens." P.N.A.S. (U.S.A.), 70: 782 - 786.

Ancri, D., Lonchampt, M. and Basset, J. (1977). "The effect of tin on the tissue distribution of 99m Tc-sodium pertechnetate." Radiology 124: 445 - 450.

Andoh, T. and Ide, T. (1973). Can. Res. 32: 1230 - 1235.

Arland, S., Chatt, J. and Davies, N. R. (1958). "The relative affinities of ligand atoms for acceptor molecules and ions." Q. Rev. Chem. Soc. 12: 265 - 276.

Armitage, P. (1971). Statistical Methods in Medical Research. Halsted, N.Y. pp. 433 - 434.

Bach, J. R. (1975). The mode of action of immunosuppressive agents. North-Holland Publishing Co., Amsterdam, N.Y.

Barrett, J. C., Tsutsui, T. and Ts'o, P. O. (1978). "Neoplastic transplantation induced by a direct perturbation of DNA." Nature 274: 222 - 232.

Benditt, E. P. (1977). "The origin of atherosclerosis.: Sci. Am. 236(2): 74 - 85.

Berenbaum, M. C. (1971). "Immunosuppression by platinium diamines." Br. J. Cancer 25: 208 - 211.

Berger, N. A. and Skinner, A. N. (1974). "Characterization of lymphocyte transformation induced by zinc ions." J. Cell. Biol. 61: 45 - 49.

Bertram, J. S. and Heidelberger, C. (1974). "Cell cycle dependency of oncogenic transformation induced by N-methyl-N'-nitro-N-nitrosuguanidme." Can Res. 34: 526 - 537.

Binder, B. (1979). "Preparation of a mixed-metal tin (II) - iron (III) compound from citrate media.: J. inorg. nucl. chem. 41: 257 - 258.

Biology Data Book (II) (1965). Ed. Altman, P. and Dittmer, D. S., Fed. of Amer. Soc. for Exptl. Biology, Washington, D. C.

Birnboim, H. C., Jevak, J. J. (1981). "Fluorometric method for rapid detection of DNA strand breaks in human white blood cells produced by low doses of radiation." Can. Res. in press.

Bisby, R. H., Cundall, R. B. and Davies, A. K. (1978). "Aspects of chemical damage in radiation and photo-biology.: Photochem. photobiol. 28: 825 - 837.

Blakey, B. R., Sisodia, C. S. and Mukkur, T. K. (1980). "The effect of methylmercury, tetraethyl lead and sodium arsenite of humoral immune response in mice.: Toxicol. Appl. Pharmacol. 52: 245 - 254.

Bourguignon, L.Y.W., Radar, R.L. and McMahon, J.T., (1979). J. Cell Physiol. 99: 95-100.

Boveri, T. (1914). Zur Frage der Enststehung maligner Tumoren. Tumoren. Jena: Gustav Fischer.

Boyd, C. M. and Dalrymple, G. V. (1974). Basic science principles of nuclear medicine. C. V. Mosby. St. Louis (1974). pp. 161 - 168.

Bradley, M. D., Hsu, I. C. and Harris, C. C. (1979). Relationship between SCE and mutagenicity, toxicity and DNA damage. Nature 282: 318 - 320.

Brakeslee, J. R., Yohn, D. S., Milo, G. E. and Hart, R. W. (1976) In: Procedeeings of the international meetings on comparative leukemia research. Ed. D. S. Yohn, Plenum press., N.Y., pp. 4981 - 483.

Brent, T. P. (1976)

Biochem. Biophs. Acta, 454 : 172 - 183.

Brookes, P. and Lawley, P.D. (1961). "Reaction of mono - and di-functional alkylating agents with nucleic acids."

Biochem. J. 80: 496 - 503.

Brown, R. A., Nazaric, C. M., deTirado, R. S., Castrillon, J. and Agard, E. T. (1977). "A comparison of the half-life of inorganic and organic tin in mice." Environ. Res. 13: 56 - 61.

Bryan, S.E., Simons, S.J., Vizard, D.L. and Hardy, K.J. (1976) "Interactions of mercury and copper with constitutive heterochromatin and euchromatin in vivo and in vitro." Biochem. 15: 1667 - 1676.

Burba, J.V. and Rudd, C. (1980). "Effect of tin (II) on some liver enzymes in mice" Abst. Soc. Exptl. Pharmacol. 1981.

Burns, D., Marzilli, L. (1977). "Relationship between molecular structure and biliary excretion of TC-99m Hida."
J. Nucl. Med. 18: 624 - 628.

Butzow, J.J. and Eichhorn, G.L. (1965). "Degradation of polyribonucleotides by zinc and other divalent metal ions." Biopolymers 3: 95 - 107.

Butzow, J.J. and Eichhorn, G.L. (1975). "Different susceptibility of DNA and RNA to cleavage by metal ions." Nature 254: 358 - 359.

Byrnes, J.J., Downey, K.M., Black, V.L. and So. A.G. (1976). A new mammalian DNA polymerase-delta.
Biochemistry 15: 2817 - 2823.

Campbell, T.C. (1980). "Chemical carcinogens and human risk assessment."
Fed. Proc. 39: 2467 - 2484.

Cerutti, P.A. (1975). "Excision repair of DNA base damage." Life Sciences 15: 1567 - 1575.

Chandler, J.A. (1977). X-ray microanalysis in the electron microscope. In: Practical methods in electron microscopy. Ed. Glauert, A.M. North Holland Publishing Co. PP. 454 - 470, 490 - 492.

Chesters, J.K. (1972). "The role of zinc ions on transformation of lymphocytes by PHA."
Biochem. J. 130: 133 - 139.

Chu, G.Y.H. and Tobias, R.S. (1976). "Heavy-metal-nucleotide interactions."
J. Amer. Chem. See. 98: 2641 - 2645.

Clark, P. and Eichhorn, G.L. (1974). "A predictable modification of enzyme specificity." Biochemistry 13: 5098 - 5102.

Clark, P. and Eichhorn, G.L. (1977). "Age changes in rat liver chromatin structure." Abstract 30th annual scientific meeting of the Gerontological.

Cleare, M.H., Hydes, P.C., Malerbi, B.W. and Watkins, D.M. (1978). "Anti-tumor platium complexes: relationships between chemical properties and activity." Biochimie 60: 835 - 850.

Cleaver, J.E. (1975). Methods for Studying Repair of DNA damaged by physical and chemical carcinogens. In: Methods in Cancer Res. 11: Ed. Busch, H. Academic Press, N.Y. PP. 123 - 165.

Cleaver, J.E. (1968). "Defective repair replication of DNA in XP." Nature 218: 652 - 656.

Cleaver, J.E. (1979). "DNA repair and its coupling to DNA replication in Eukaryotic cells Biochim. Biophys. Acta. 516: 489 - 516.

Cooper, J.R., Stradling, N.G., Smith, H. and Ham., S.E. (1980). "The reactions of 1nm. particles of plutonium 238 dioxide and curium dioxide with lung fluid."

Int. J. Radiat. Biol. 38: 93 - 103.

Cloutier, R.J., Coffey, J.L., Snyder, W.S. and Watson, E.E. (1976)

Radiopharmaceutical Dosimetry Symposium

Oak Ridge, Tenn. 4.29.76

U.S.D.H.E.W., Rockville, Md.

Corry, P.M. and Cole, A. (1973) "Double strand rejoining in mammalian DNA. Nature 245: 100 - 101.

Costa, M. (1979). Metal Carcinogenesis Testing. Humana Press. Cliftron, N.J. PP 111 - 125.

CRC Handbook of Radioactive Nuclides. (1969) Ed. Wang, Y. The chemical Rubber Company, Ohio. PP. 77 - 85.

Craddock, V.M. (1975). "Effect of a single treatment with the alkylating carcinogens dimethylnitrosamine, diethylnitrosamine and methyl methonesulfonate, on liver regeneration after partial hepatectomy." Chem. Biol. Interact. 10: 323 - 332.

Crapper, D.R., Krishnan, S.S. and Quittkat, S. (1976). "Aluminium, neurofibrillary degeneration and Alzheimer's disease. Brain 99; 67 - 80.

Cremer, J. (1970). "Selective inhibition of glucose oxidation by triethyltin in rat brain in vivo." Biochem. J. 119: 95 - 102.

Crowe, A.J., Smith, P.J. and Atassi, G. (1980). "Investigations into the antitumor activity of Organotin Compounds, I. Dioganotin dihalide and di-pseudohalide complexes." Chem. Biol. Interact. 32: 171 - 178.

Cunningham,A.J. and Szenberg,A. (1968)."Further improvements in the plaque technique for determining single antibody forming cells." Immunol. 14,599-601.

Dahl, A.R. and Hodgson, E. (1977). "Complexes of stannous fluoride and other group IV B dihalides with mammalian hemoproteins." Science 197: 1376 - 1378.

d'Ambrosio, S.M. and Setlow, R.B. (1976). "Enhancement of postreplication repair in Chinese hamster cells." P.N.A.S. 73: 2396 - 2400.

Dean, J.H., Padarathsingh, M.L. and Jerrells, T.R. (1979). "Application of immunocompetence assays for defining immunosuppression. In: Health effects of halogenated aromatic hydrocarbons." Ed: Nicholson, W.J. and Moore, J.A. N.Y. Acad. Sci. 320: 579 - 590.

DeKnudt, G., Leonard, A. and Ivanoz, B. (1973). Chromosome aberrations observed in male workers occupationally exposed to lead." Environ. physio. Biochem. 3: 132 - 138.

Deutsch, E., Elder, R.C., and Lange, B.A. (1976). Structural characterizaton of a bridged 99m Tc-Sn-dimethylglyoxime complex." P.N.A.S. 73: 4287 - 4289.

Dizikes, L.J., Ridley, W.P. and Wood, J.M. (1978). "A mechanism for biomethylation of tin by reductive Co-C bond cleavage of alkylcobalamins." J. Am. Chem. Soc. 100: 1010 - 1012.

Doniger, J. and Grossman, L. (1976). Human correxonuclease J. Biol. Chem. 251: 4579 - 4587.

Douglas, G., and Grant, C.E. (1980). "DNA damage, cell survival and DNA repair in chinese hamster ovary cells after in in vitro metabolcc activation of dimethylnitrosamine andd benzo(a)pyrene." Mutat. Res.

Duker, N.J. and Teebor, G.W. (1976). "Detection of different types of damage in alkylated DNA by means of human corrective endonuclease (correndonuclease)." PNAS (U.S.A.) 73: 2629 - 2633.

Dutton, R.W. and Mishell, R.I. (1967). "Cell populations and cell proliferation in the vitro response of normal mouse spleen to heterologous erythrocytes." J. Exptl. Med. 126: 443 - 455.

Eckelman, W.C., Smith, T.D. andd Richards, P. "Labeling blood cells with 99mTc." In: Radiopharmaceuticals. Eds. Subramanian, G., Rhodes, B.A., Cooper, J.F. and Sodd, V.J. Soc. Nucl. Med. NY PP. 36 - 49.

Eichhorn, G.L., Shin, Y.A., Clark, P., Rifkind, J., Pitha, J. et al. (1979).

Essential and deleterious effects in the interaction of metal ions with nucleic acids. In: Trace metals in health and disease. Ed. Kharasch, N. Raven Press, N.Y. (1979), PP. 123 - 133.

Evans, H.J. and O'Riordan, M.L. (1975). "Human peripheral blood lymphocytes for the analysis of chromosome aberrations in mutagen tests." Mutat. Res. 31: 135 - 148.

Festing, M. F. W. ed. (1975) International index of laboratory animals. Animal Laboratory, Animal Center. Carshalton, Surrey, Engl.

Flesse, C. P. (1979). Metals as mutagenic initiators of cancer. In: Trace metals in health and disease. Ed. Kharasch, N., Raven Press, N.Y., pp. 109 - 1023.

Freund, J. E. and Williams, F. J. (1958). Modern Statistics. Prentice Hall N. J., pp. 187 - 244.

Friedberg, E. C., Cook, K. H., Duncan, J. and Mortelmans, K. (1977). "DNA repair enzymes in mammalian cells." In: Photochem. Photobiol. Rev. 2: Ed. Smith, K. L., Plenum Press, N.Y.

Furchner, J. E. and Drake G. A. (1976). "Comparative metabolism of radionuclides in mammals X 1. Retention of 113 Tin (II) in the mouse, rat, monkey and dog. Health Phys. 31: 219 - 224.

Furst, A. (1977). "Inorganic agents as carcinogens." In: Advances in modern toxicoloty, vol. 3. Eds: Kraybill, H. and Mehlman, M. Wuketm N. Y. 1979, pp. 1 - 12.

Gaworski, C. L. and Sharma, R. P. (1978). "The effects of heavy metals on ^{3}H-thymidine uptake in lymphocytes." Toxicol. Appl. Pharmacol. 46: 305 - 313.

Geraci, J. P., Christensen, G. M. and Jackson, K. L. (1975). "A comparison of the radiation sensitivities of thymic and thoracic duct lymphocytes." Radiat. Res. 61: 158 - 163.

Glauert, A. M. (1975). Fixation, dehydration and embedding of biological specimens. In: Practical methods in electron microscopy. Ed. Glavert, A. M., North-Holland Publishing Col

Goh, K., Reddy, M. and Hempelmann, L. H. (1976). "Chromosomal aberrations in lymphocytes of normal adults long after thymus irradiation." Radiat. Res. 67: 82 - 85.

Goth, R. and Rajewsky, M. F. (1974). "persistence of 06-ethylguanine in rat brain DNA: correlation with nervous system-specific carcinogenesis by ethylnitrosourea." P.N.A.S. 71: 639 - 643.

Gray, I. (1977). "Trace metal modification of immunocompetence. Cellular immunology." 34: 19 - 24.

Greaves, M. F., Owen, J. J. T. and Raff, M. C. T and B Lymphocytes: origins, properties and roles in immune resposes, Excerpta Medica, Amsterdam, 19[illegible].

Grossman, L. (1974). Advances in radiation biology 4: 77.

Gurley, L. R., Walters, R. A., Jett, J. H. and Tobey, R. A. (1980). "Respon[illegible] of CHO cell proliferation and histone phosphorylation to sodium arsenite." J. Toxicol. Environ. Health 6: 87 - 105.

Hadley, J. G., Conklin, A. W. and Saunders, C. L. (1980). "Rapid solubilization and translocation of 109 CdO following pulmonary deposition." Toxicol. Appl. Pharmacol. 54: 156 - 160.

Hafeman, D. G. and Lucan, Z. J. (1979). "Polymorphonuclear leukocyte mediated antibody dependent cellular cytotoxicity against tumor cells: dependence on oxygen and the respiratory burst." J. of Immunology. 123: 55 - 62.

Hamilton, E. I., Minski, M. J. and Cleary, J. J. (1972/73). Toxic metals and their analysis. Sci. Total Environ. 1, 341 - 346.

Hand, R. (1978). "Prereplicative error-free DNA repair." Biochem. Pharmacol. 29: 977 - 982.

Hart, R.W., Hall, K.Y. and Daniel, B. "DNA Repair and Mutagenesis in Mammalian Cells." Photochem. Photobiol. 28: 131 - 155.

Hassan, F. M. and Kazemi, H. (1974). "Chronic beryllium disease:" chest 65: 289.

Hazelton, G. A. and Lang, C. A. (1980). "Glutathione contents of tissues in the aging mouse." Biochem. J. 188: 25 - 30.

Heidelberger, C. (1975). "Chemical carcinogenesis." Ann. Rev. Biochem 44: 79 - 21.

Heilbrunn, L. V. (1952). An outline of general physiology. W. B. Saunders, Philadelphia. pp. 498 - 502.

Hemminki, K., Saloniemi, I., Luoma, K., Salonen, T., Partanen, T. et al. (1979) "Transplacental carcinogens and mutagens:" J. Toxicol. Environ. Health 6: 1115 - 1126.

Henry, R. A. and Byington, K. H. (1976). "Inhibition of glutathione-s-aryltransferase from rat liver by organomercury, lead and tin compounds." Biochem Pharmacol. 25: 2291.

Higginson, J. (1976). "Importance of environmental factors in cancer." NSERN Symposia. 52: 15 - 23.

Higginson, J. (1976). "A hazardous society? Individual vs. community responsibility in cancer prevention." Am. J. Public Health 55: 359 - 366.

Hiles, R. A. (1974). "Absorption, distribution and excretion of inorganic tin in rats." Toxicol. Appl. Pharmacol. 27: 366 - 379.

Hodge, V. F., Seidel, S. L. and Goldberg, E. D. (1979). "Determination of Sn(IV) and organotin compounds in natural waters, coastal sediments and macro algae by atomic absorption spectrometry." Anal. Chem. 51: 1256 - 1259.

Huberman, E. and Sachs, L. (1974). "Cell mediated mutagenesis of mammalian cells with chemical carcinogens." Int. J. Cancer 13: 326 - 333.

Hudson, R. J., Saben, H. S. and Emslie, D. (1974). "Physiological and environmental influences in immunity." Vet. Bull. 44: 119.

Huey, C., Brinkman, R. E., Grim, S. and Iverson, W. P. (1974). In: Proceedings of the international conference on transport of persistent chemicals in aquatic ecosystems. Ed. Laham, Q. N. NRC, Canada, Ottawa. pp. 73 - 78.

Hutchinson, E. (1959). Chemistry: The elements and their reactions. W. B. Saunders Co. Phil. and N.Y. pp. 325 - 335.

Humphrey, R. M., Stewart, D. L. and Sedita, B. A. (1968). "DNA-strand breaks and rejoining following exposure of synchronized chinese hamster cells." Nutat. Res. 6: 459 - 462.

IARC. (1972). Monographs on the evaluation of carcinogenic risk to man. Vol. 1, pp. 40 - 53. Lyon: International Agency for research in cancer.

Jackson, K. L., Christensen, G. M. and Bistline, R. W. (1969). "Irreparable injury produced by x-irradiation of rat thymocytes in vitro" Radiat. Res. 38: 560 - 568.

Jelen, A. and Lindquist, O. (1969). "The Crystal Structure of Potassium Triformatostannate (II). Acta Chemica Scandinavica" 23: 3071 - 3080.

Kampf, G., Tolkendorf, E., Regel, K. and Abel, H. (1977). "Cell inactivation and DNA strand break rates after irradiation with x-rays and fast neutrons." Studia Biophysica. 62: 17 - 24.

Kaplan, J. C., Kushner, R. and Grossman, L. (1969). "enzymatic repair of DNA I." P.N.A.S. 63: 144 - 151.

Kappas, A. and Maines, M. D. (1976). Tin: A potent inducer of heme oxygenase in kidney. Science. 192: 60 - 62.

Kay, N. E., Bumol, T. F. and Douglas, S. D. (1980). "Effects of 2-DOG on human monocyte metabolism." J. of Reticuloendotrhel. Soc. 28: 367 - 379.

Kehoe, R. A., Cholak, J. and Story, R. V. (1940). "A spectrophotometrical study of the normal ranges of concentration of certain trace metals in biological materials." J. Nutr. 19: 579 - 587.

Keilin, D. (1929). "Cytochrome and respiratory enzymes." Proc. R. Soc. Lond. (Biol.) 104: 206 - 252.

Kennedy, A. R. and Little, J. B. (1978). Nature 276: 825 - 826.

Kent, N. L. and McCance, V. R. A. (1941). "The absorption and excretion of minor elements by man: nickel, cobalt, tin and manganese." Biochem. J. 35: 877 - 883.

Khan-van-kien, L. and Tuong, T. (1956). "Histochemical observation of the fixation and elimination of tin compounds by livers of rats and rabbits in relation to the mode of administration." Soc. Biol. Montpellier 150: 2230 - 2233.

Khentigan, A., Garrett, M., Lum, D. and Winchell, H. S. (1976). "Effects of prior administration of Sn (II) complexes on in vivo distribution of 99m Tc-pertechnetate." J. Nucl. Med. 17: 480 - 384.

Kinsella, A. and Radman, M. (1978). P.N.A.S. 25: 6149 - 6153.

Knudson, A. G. (1977). "Genetics and etiology of human Cancer." Adv. Hum. Genet. 8: 1 - 66.

Kohn, K. W. (1979). "DNA as a target in Cancer chemotherapy:" Methods in Cancer Res. 16: 291 - 343.

Koller, L. D. (1973). "Immunosuppression produced by lead, cadmium and mercury.: Amer. J. Vet. Res. 34: 1457 - 1458.

Kutz, S. A., Hinsdill, R. D. and Weltman, D. J. (1980). "Evaluation of chemicals for immunomodulatory effects using an in-vitro antibody-producing assay." Environ. Res. 22: 368 - 376.

Langer, G. and Bogucki, R. F. (1967). "The chemistry of tin (II) chelates - II." J. inorg. nucl. chem. 29: 495 - 502.

Langewoort, H. L., Cohn, Z. A., Hirsch, J. G., Humphrey, H. H. et al. (1970). "The nomenchlature of mononuclear phagocytic cells." Ed. Van Furth, R. Blackwell Scientific, Oxford, 1970. pp. 1 - 20.

Laval, F. (1980). "Effect of uncouplers on radiosensitivity and mutagenicity in x-irradiated mammalian cells. P.N.A.S. 77: 2702 - 2705.

Lavrukhina, A.K., Malysheva, T.V. and Pavlotskaya, F.J. (1967). "Chemical analysis of radioactive materials." CRC Press, Cleveland, U.S.A., 10-150.

Lawrence, D. A. (1981). Heavy metal modulation of lymphocyte activities. Tocicol. Appl. Pharmacol. 57, 439 - 451.

Leenhouts, H. P. and Chadwick, K. H. (1978). "The crucial role of DNA double-strand breaks in cellular radiobiological effects." In: Advances in Radiation Biology 7: 55 - 101.

Lett, J. T., Caldwell, I., Dean, C. J. and Alexander, P. (1967). "Rejoining of x-ray induced bvreaks in the DNA." Nature 214: 790 - 792.

Levis, A., Buttignol, M., Bianchi, V. and Sponza, G. (1978). "Effects of potassium dichromate on nucleic acid and protein synthesis and on precursor uptake in BHK fibroblasts." Cancer. Res. 38: 110 - 116.

Lewis, J. G. and Swenberg, J. A. (1980). "Differential repair of 06-methylquanine in DNA of rat hepatocytes and nonparenchymal cells. Nature: 288: 186 - 187.

Lehmann, A. R. and Ormerod, M. G. (1970). "The replication of DNA in murine lymphoma cells (L517894)." Biochem. Biophys. Acta 204: 128 - 143.

Lehmann, A. R. (1972). "Minireview: Postreplication repair in mammalian cells." Life Science 15: 2005 - 2016.

Lindahl, T. (1976). "New class of enzymes acting on DNA." Nature 259: 64 - 66.

Lin, M. S. (1974). "Labeling proteins with 99m Tc. In: radiopharmaceuticals." Eds: Subramanian, G., Rhodes, B. A., Cooper, J. F. and Sodd, V. J. Society of Nuclear Medicine, N. Y. pp. 36 - 49.

Littlefield, L. G., Calyer, S. P., Joiner, E. E. and Dugrain, R. J. (1979). "Sister chromated exchange in human lymphocytes exposed to ionizing radiation during Go." Radiat. Res. 78: 514 - 521.

Lloyd, R. V. and Rogers, M. T. (1973). "E. S. R. study of some silicon, germanium and tin-centered radicals." J. Amer. Chem. Soc. 95: 2459 - 2464.

Loewenstein, W. (1966). "Permeability of membrane junctions." Conference on biological membranes. In: Recent progress. Ann. N. Y. Acad. Sci. 137: 441 - 447.

Loprieno, N. (1977). "Occupational Hazards: Environmental Mutagens." Akademie-Verlag, Berlin 1977, pp. 153 - 160.

Lutz, W. (1967). Statistical Methods as Applied to Immunological Data Handbook of Experimental Immunology. Ed. Weir, D. M. Blackwell Publications, Oxford. pp. 1164 - 1206.

Magee, P. N., Nicholl, J. W., Pegg, A. E. and Swann, P. F. (1975). "Alkylating intermediates in nitrosamine metabolism." Biochem. Soc. Trans. 3: 62 - 65.

Mahler, H. R. and Cordes, E. H. (1966). Biological Chemistry. Harper Row, N. Y pp. 394 - 397.

Mahler, H. R. and Cordes, E. H. (1966a). Biological Chemistry. Harper Row, N. Y pp. 267 - 277.

Maines, M. D. and Kappas, A. (1977)a. "Metals as regulators of heme metabolism." Science 198: 1215 - 1221.

Maines, M. D. and Kappas, A. (1977)b. "Regulation of heme pathway enzymes and cellular glutathione content by metals that do not chelate with tetrapyrroles." P.N.A.S. 74: 1875 - 1878.

Mak, K. M. and Chang, W. W. (1978). "Inhibition of DNA synthesis by 1, 2-dimethylhydrazine and methylazoxomethanol acetate in rabbit colon mucosa in organ culture." J.N.C.I. 61: 799 - 805.

Malz, W., Hartwig, M., Korner, I. J., Handschack and Rakow, A. (1975). "DNA lesions and their repair in radiation damaged cultures of mamallian cells." 53: 13 - 15.

Marquardt, H., Philips, F. S. and Bendich, A. (1972). "DNA binding and inhibition of DNA synthesis after 7, 12 dimethylbenz(a)anthracene administered during the early prepeplicative phase in regenerating rat liver." Cancer Res. 32 1810 - 1813.

Maugh, T. H. (1978). "Chemicals: How many are there?" Science. 199: 162 - 166.

Maugh, T. H. (1978). "Chemical carcinogens." Science 201: 1200 - 1205.

McGrath, R. A. and Williams, R. W. (1966). "Reconstruction in vivo of irradiated E. Coli deoxyribonucleic acid: the rejoining of broken pieces." Nature 212: 534 - 535.

McLean,J.R. and Kaplan, J.G. (1979). "The effect of tin on unscheduled and semi-conservative DNA synthesis" in "The molecular basis of immune cell function" ed J.G. Kaplan, Elsevier/North-Holland Press Amsterdam,pp412-416.

McLean,J.R., McWilliams,R.S., kaplan,J.G.and Birnboim,H.C. (1981). "Rapid detection of DNA strand breaks in human peripheral blood and animal organs following treatment with physical and chemical agents." in press.

McRae, J., Sugar, R. M., Shipley, B. and Hook, G. R. (1974). "Alterations in tissue distribution of 99m Tc-pertechnetate in rats given stannous tin." J. Nucl. Med. 15: 151 - 155.

Metzger, G., Wilhelm, F. X. and Wilhelm, M. L. (1977). "Non-random binding of a chemical carcinogen to DNA in chromatin" Biochem. Biophys. Res. Commun. 75: 703 - 710.

Michelson, A. M. (1977). "Production of superoxide by metal ions." In: Super oxide and superoxide dismutases. Eds: Michelson, A. M., McCord, J. M.

Mirvisch, S. S., Chu, C. and Clayson, D. B. (1978). "Inhibition of 3H thymidine incorporation into DNA of rat esophageal epithelium and related tissues by carcinogenic N-nitroso compounds." Cancer res. 38: 305 - 310.

Mischel, R. I. and Dutton, R. W. (1967). "Immunization of dissociated spleen cell cultures from normal mice.: J. Exptl. Med. 126: 423 - 442.

Mondal, S. and Heidelberger, C. (1970). "In vitro malignant transformation by methylcholanthrene of the progeny of single cells derived from C3H mouse prostate." P.N.A.S. 65: 219 - 229.

More, K. E. and Brody, T. M. (1961). "The effect of triethyltin on tissue amines." J. Pharmacol. Exp. Ther. 132: 6 - 12.

Morse, S., Stearns, C. D. and Goldsmith, S. R. (1975). "Lymphatic depletion induced by cholera toxin." J. Immunol. 114: 665 - 669.

Nardelli, M., Pelizzi, C. and Pelizzi, G. (1975). "Tin complexes containing the nitrate group:" J. Chem. Soc. Dalton Trans. 1595 - 1597.

Nelp, W. B. (1969). "Distribution and Radiological Behavior of colloids and macroaggregates." In: Medical Radionuclides: Radiation Dose and Effects. Ed. Cloutier, R. J., Edwards, C. L. and Snyder, W. S. U.S.A.E.C., Oak Ridge, Tenn. pp. 239 - 153.

Nichols, W. W. (1975). "Somatic mutation in biological research." Hereditas 81: 225 - 236.

Nieboir, E. and Richardson, D. H. S. (1980). "The replacement of nondescript term 'heavy metal' by a biologically and chemically significant classification of metal ions." Environ. Pollution (Ser. B) 1: 3 - 26.

Nielsen, F. H. and Sandstead, H. H. (1974). "Are nickel, vanadium, silicon, fluorine and tin essential for man? A review." Am. J. Clin. Hutr. 27: 515 - 520.

Norseth, T. (1978). "Asbestos and metals as carcinogens." J. Environ. Pathol. Toxicol. 2: 1021 - 1028.

Nossal, G. J. V., Austin, C. M., Pye, J. and Mitchell, J. (1966). "Antigens in Immunity." Int. Arch. Allergy 29: 368 - 383.

O'Connor, P. J., Capps, M. J. and Craig, A. W. (1973). Comparative studies of the hepatocarcinogen N-N dimethylnitrosamine in vivo" Br. J. Cancer. 27: 153 - 166.

Ormerod, M. G. and Lehmann, A. R. (1971). "Artifacts arising from the sedimentation of high molecular weight DNA on sucrose gradients." Biochem. Biophys. Acta. 247: 369 - 372.

Ormerod, M. G. (1977). "Radiation-induced strand breaks in the DNA of mammalian cells." In: Biology of Radiation Carcinogenesis. Eds: Yuhas, J. M., Tennant, R. W. and Regen, J. D., Raven Press, N. Y. pp. 67 - 93.

Ormos, G., Manyai, S. (1977). "Chemical modification of erythrocytes. Effect on the velocity of chromate uptake. Acta Biochim et Biophys. 12: 343 - 352.

Oskarsson, A., Andersson, Y and Tjalve, H. (1979). "Fate of nickel subsulfide during carcinogenesis studied by autoradiography and x-ray powder diffraction." Cancer. Res. 39: 4175 - 4182.

Painter, R. B. and Young, B. R. (1972). "Repair replication in mammalian cells after x-irradiation." Mutat. Res. 14: 225 - 235.

Painter, R. B. (1975). "Chemical changes induced in DNA by ionizing radiation and the relationship of their repair to survival of mamalian cells." In: Radiation Research, Eds.: Nygaard, O., Adler, H. I. and Sinclair, W. K., Academic Press, N. Y. pp. 735 - 739.

Painter, R. B. (1977). "Rapid test to detect agents that damage human DNA." Nature 265: 650 - 651.

Painter, R. B. (1978). "DNA synthesis inhibition in He La cells as a simple test for agents that damage human DNA." J. Environ. Path. Toxicol. 2: 65 - 78.

Painter, R. B. (1980). "A replication model for sister-chromated exchange." Mutat. Res, 70: 337 - 341.

Palcic, B. and Skarsgard, D. (1972). "The effect of Oxygen on DNA single-strand breaks produced by ionizing radiation in Mamalian cells." Int. J. Radiat. Biol. 21: 417 - 433.

Parodi, S. and Brombella, G. (1977). "Relationship between mutation and transformation frequencies in mammalian cells." Int. J. Radiat. Biol. 21: 417 - 433.

Patel, M. C., Parab, P. B., Samual, A. M. and Ganatra, R. D., (1977). "Labelling of red blod cells with 99mTc after oral administration of $SnCl_2$:" J. Nucl. Med. 20: 877 - 881.

Paterson, M. C., Smith, B. P., Lohman, P. H. M., Anderson, A. K. and Fishman, L. (1976). "Defective excision repair of x-ray damaged DNA in human (A.T.) fiboblasts." Nature 260: 444 - 447.

Pearson, R. G. (1956). "Hard and soft acids and bases." J. Amer. Chem. Soc. 85: 3533 - 3539.

Pearson, R. G. (1968). "Hard and soft acids and bases, HSAB Part I. Fundamental Principals." J. Chem. Educ. 45: 581 - 587.

Pearson, R. G. (1968a). "Hard and soft acids and bases, HSAB, Part II. Underlying Theories." J. Chem. Educ. 45: 643 - 648.

Pearson, R. G. (1969). "Hard and soft acids and bases." Surv. Prog. Chem. 5: 1 - 52.

Perry, P. E. (1979). "Chemical mutagens and sister chromated exchange." In: Chemical mutagens, principles and methods for their detection. Volume 6. Eds: deSerres, F. J. and Hollaender, A. Plenum Press, N. Y. pp. 1 - 39.

Plagemann, P. G. and Richey, D. P. (1974). "Transport of nucleosides, nucleic acid bases, choline and glucose by animal cells in culture." Biochim. Biophys. Acta. 334: 263 - 305.

Povirk, L. F. and Painter, R. B. (1976). "The effect of 313 nm light on initiation of replicons in mammalian cell DNA containing bromodeoxyuridine." Biochim. Biophys. Acta. 432: 267 - 272.

Pratten, M. K., Williams, K. E. and Lloyd, J. B. (1977). A Quantitative study of pinocytosis and intracellular proteolysis in rat peritoneal macrophages. Biochem. J. 168, 365 - 372.

Pryor, W. A. (1978). "The formation of free radicals and the consequences of their reactions in vivo." Photochem. Photobiol. 28: 787 - 801.

Quagliano, J. V. and Vallarino, L. M. (1963a). Chemistry, 3rd edition. Prentice-Hall Inc. Englewood Cliffs, N. J. pp. 824 - 825(a), 1[illegible] (b).

Rahman, Y. E. (1979). "Potential of the liposomal approach to metal chelation therapy." In: Lysosomes, V. 6, Eds. Dingle, J. T., Jacques, P. J. and Shaw, I. H. North-Holland Publishing Co. Amsterdam, pp. 625 - 652.

Regan, J. D. and Setlow, R. B. (1974). "Two forms of repair in the DNA of human cells damaged by chemical carcinogens and mutagens." Cancer Res. 34: 3318 - 3325.

Reiner, E. (1971). "Binding of Be to protein." In: A symposium on mechanisms of toxicity. Ed: Aldridge, W. N. MacMillan, London, pp. 111 - 125.

Resnick, M. A. 91976). "The repair of double-strand breaks in DNA: a model involving recombination." J. Theoret. Biol. 59: 97 - 103.

Reznikoff, C., Bertram, J., Brankoq, D. and Heidelberger, C. (1973). "Quantitative and qualitative studies of chemical transformation of cloned C3H mouse embryo cells sensitive to postconfluence inhibition of cell division." Can. Res. 33: 3239 - 3249.

Ridley, W. P., Dizikes, L. J. and Wood, J. M. (1977). "Biomethylation of toxic elements in the environment." Science 197: 329 - 332.

Roberts, J. J. and Thomson, A. J. (1979). "The mechanism of action of antitumor platinum compounds.:" Prog. Nucl. Ac. Res. and Mol. Biol. 22: 71 - 133.

Robinson, I. M. (1969). F. Cosmet. Toxic 7: 47 - 52.

Rodman, M. (1975). In: Molecular mechanisms for repair of DNA. Eds: Honawalt, P. and Setlow, R. B. Plenum Press, N. Y. pp. 355 - 367.

Rosenstreich, D. L. and Mizel, S. B. (1978). "The participation of macrophages and macrophage cell lines in the activation of T-lymphocytes by mitogen." Immunological Rev. 40: 102 - 104.

Rosenthal, A. S., Moses, H. L., Beaver, D. L. and Schuffman, S. S. (1966). "Lead ion and phosphatase histochemistry. I. Nonenzymatic hydrolysis of nucleoside phosphates by lead ion." J. Of Histochem. Cytochem. 14: 698 - 701.

Rowley, J. (1977). "A possible role for nonrandom chromosomal changes in human hematologic malignancies." In: Chromosomes today. Eds: de la Chapelle, A., and Sorsa, M. Amsterdam Elsevier (North-Holand). pp. 345 - 355.

Rubin, H. (1976). "Carcinogenicity test.:" Science, 191: 241.

Rubin, H. (1980). "Is somatic mutation the major mechanism of malignant transformation?" JNCI. 64: 995 - 1001.

Rupp, W. D. and Howard-Flanders, P. (1968). Discontinuities in the DNA synthesized in an excision defective strain of E. Coli following U.V. irradiation. J. Mol. Biol. 31: 291 - 304.

Saba, T. M. (1970). "Structure and Function of the Reticuloendothelial System." Arch. Int. Med. 126: 1030 - 1050.

San, R. H. C. and Stich, H. F. (1975). "DNA repair synthesis of cultured human cells as a rapid bioassay for chemical carcinogens." Int. J. Cancer. 16: 284 - 291.

Sarasin, A. (1978). "Induced DNA repair processes in eucaryotic cells." Biochimie 60: 1141 - 1144.

Schroeder, H. A., Balassa, J. J. and Tipton, I. H. (1964). "Abnormal trace elements in man: tin." J. Chronic Dis. 17: 483 - 502.

Schroeder, H. A. (1965). "The biological trace elements or peripatetics through the periodic table." J. Chem. Dis. 18: 217.

Schwarz, K., Milne, D. B. and Vinyard, M. (1970). "Growth effects of tin compounds in rats maintained in a trace-element controlled environment." Biochem Biophys. Res. Commun. 40: 22 - 29.

Schwarz, K. (1974). "New essential trace elements (Sn, V, F, Si): Progress report and outlook." In: Tracé element metabolism in animals - 2. Eds: Hoekstra, W. G., Suttie, J. W., Ganther, H. E. and Mertz, W., University Park Press, Baltimore, pp. 355 - 377.

Seger, N., Miller, C., Sharon, R. and Ben-Ishai, R. (1973). "Excision repair of UV radiation damage in toluene treated E. Coli." Biochem. Biophys. Res. Commun. 52: 1241 - 1245.

Seiler J. P. (1977). "Inhibition of testicular DNA synthesis by chemical mutagens and carcinogens." Mutat. Res. 46: 305 - 310.

Seinen, W., Vos, J. G., Brands, R. and Hooykaas, H. (1979). "Lymphocytoxicity and immunosuppression by organotin compounds." Immunopharmacology 1: 343 - 356.

Sharif, A., Jacquet, M. A., Simonnet, G. and Engler, R. (1980). "A simple and economical procedure for liquid scintillation counting of tritiated compounds on glass fiber filters.: Int. J. Appl. Radiat. and Isot. 31: 665 - 671.

Shenker, B. J., Matarazzo, W. J., Hirsch, R. L. and Gray, I. (1977). Trace metal modification of immunocompetence. Cellular immunology 34: 19 - 24.

Sillen, L. G. and Martell, A. E. (1964). Stability constants of metal-ion complexes. The Chem. Soc. (London) special public. 17.

Sillén, L. B. and Martell, A. E. (1974). Stability constants of metal-ion complexes, suppl. 1. The Chem. Soc. (London) special public. 25.

Silva, M. and Valcarcel, M. (1980). "Extraction and spectrophometric determination of trace amounts of tin (II) with diphenylglyoxal." Microchem, J. 25: 289 - 295.

Simmon, V. (1979). "In vitro mutagenicity assays of chemical carcinogens and related compounds with Salmonella typhimumium." J.N.C.I. 62: 893 - 899.

Simpson, I., Rose, B., and Loewenstein, W. (1977). "Size limit of molecules permeating the junctional membrane channels." Science 195: 294 - 296.

Singh, A. (1978), "Introduction: Interconversion of singlet oxygen and related species." Photochem. Photobiol. 28: 429 - 433.

Sirover, M. A. and Loeb, L. A. (1976). "Infidelity of DNA synthesis in-vitro: Screening for potential metal mutagens or carcinogens."

Sivak, A. (1976) "The Ames test." Science 193: 272 - 273.

Smith, T. D. (1961). "The chelates formed by Tin (II) with certain Amino-poly carboxylic acids." J. Chem. Soc. 2554 - 2557.

Stenstrand, K., Toivonen, H. and Rytomaa, T. (1979). "Radiation-induced chromosome abberrations in human lymphocytes." Publication of the Institute of Radiation Protection, Helsinki, publication. STL-A29.

Stradling, G. N., Cooper, J. R., Smith, H. and Ham, S. E. (1979). "The mobility of curium-244 dioxide in the bronchially intubated rat." Int. J. Radiat. Biol. 36: 19 - 32.

Strauss, P. R., Sheehan, J. M. and Kashket, E. R. (1977). "Membrane transport by murine lymphocytes." Immunology 118: 1328 - 1334.

Sunderman, F. (1978). "Carcinogenic effects of metals." Fed. Proc. 37: 76 - 79.

Sunderman, F. W. (1978a). "Carcinogenic effects of metals." Fed. Proc. 37: 40 - 46.

Tauber, A. I. and Babior, B. M. (1978). "O_2 and host defense: The production and fate of O_2 in neutrophils." Photochem. Photobiol. 28: 701 - 709.

Thompson, A. J., Williams, R. J. P. and Reslova, A. "The chemistry of complexes relating to CIS-Pt $(NH_3)_2Cl_{2s}$: an antitumor drug." Struct. Bonding (berl.) 11: 1 - 64.

Tsan, M. F. and Denison, R. C. (1980) "PMA-induced neutrophil autotoxicity." Inflammation, 4: 371 - 380.

Tyzzer, E. E. (1916). "Tumor immunity." J. Cancer Res. 1: 125.

van den Berg, J. M. (1961). "The crystal structure of $SnCl_2$." Acta Cryst. 14: 1002 - 1003.

van Dijk, H., Bakker, J. A., Testerink, J. et al. (1975) "Oxisuran and immune reactions." J. Immunol. 115: 1587 - 1596.

van Furth, R. (1975). "The effect of immunosuppressive drugs on the formation and kinetics of mononuclear phagocytes." In: Activation of macrophages, proc. 2nd Workshop Conference, Hoechst, Eds: Wagner, W. H. and Hahn, H. Excerpta Medica, Amsterdam, P. 178.

van Remoortere, F. P., Flynn, J. J. and Boer, F. P. (1971a)., "The crystal structure of stannic EDTA monohydrate." Inorg. Chem. 10: 2313 - 1219.

van Remoortere, F. P., Flynn, J. J., Boer, F. P. and North, P. P. (1971b). "The crystal structure of distannous EDTH dihydrate." Inorg. Chem. 10: 1511 - 1518.

Venitt, S. and Levy, L. S. (1974). "Mutagenicity of chromates in bacteria and its relevance to chromate carcinogenesis." Nature 250: 493 - 495.

Verly, W. G. (1980). "Prereplicative error-free DNA repair." Biochem. Pharmacol. 29: 977 - 982.

von Hahn, H. P. Miller, H. P. and Eichhorn, G. L. (1969). "Age-related alternations in the structure of nucleoprotein IV." Gerontologia 15: 293 - 301.

von Hahn, H. P. Miller, H. P. and Eichhorn, G. L. (1970). "The effect of divalent ions on the isolation of proteins from rat liver nucleoproteins." Biochim Biophys. Acta 214: 509 - 519.

Vos, J. G. (1977). "Immune suppression as related to toxicology." CRC in Toxicology 5: 67 - 107.

Wade, M. H., Chu, E. H. Y and Schmickel, R. D. (1977). "U.V. light sensitivity and deficiency in DNA polymerase activity in cultured skin fibroblasts from patients with Cockayne Syndrome." Mutat. Res. 46: 162.

Walker, I. C. and Ewart, D. F. (1973). "The nature of single-strand breaks in DNA following treatment of L-cells with methylating agents." Mutat. Res. 19: 331 - 341.

Warren, J. R. and Summerville, J. W. (1980). "Activity of carcinogens, carcinogen analogs and metabolic inhibitors in the lymphocyte tritiated thymidine assay." JNCI 65: 1321 - 1328.

Williams, J. R., Little, J. B. and Shipley, W. V. (1974). "Association of cell death with specific endonucleolytic degradation of DNA". Nature 252:754-755.

Williams, J. R. (1978). "Role of DNA Repair in Cell Inactivation, Aging, and Transformation: A Selective Review, A Speculative Model."

Adv. Radiat. Biol. 6 : 162-207.

Witkin, E. (1976). UV mutagenesis and inducible DNA repair in E. Coli."

Bacteriol. Rev. 40: 869 - 907.

Wold, F. (1971). Macromolecules: Structure and Function. Prentice Hall N.J.

pp. 16 - 52.

Wolff, S. (1979). "Sister chromated exchange: the most sensitive mammalian system for determining the effects of mutagenic carcinogens." In: Genetic damage in man caused by environmental agents. Ed: Berg, K.

Acad. Press. pp. 229 - 246.

Wood, J. M. (1974). "Biological cycles for toxic elements in the environment.: Science 183: 1049 - 1052.

Yamaguchi, M., Suzuki, H. and Yamamoto, T. (1978a). "Suppression of Insulin secretion by oral administration of stannous chloride." Toxicol. Lett. 2: 111 - 114.

Yamaguchi, M., Suzuki, H. and Yamamoto, T. (1978b). "Decrease of phosphorylase activity in the liver of rats orally administered stannous chloride."

Toxicol. Lett. 2: 171 - 174.

Yamaguchi, M., and Yamamoto, T. (1978c). "Effect of tin on calcium content in bile of rats." Toxicol. Appl. Pharmacol. 45: 611 - 616.

Yamaguchi, M., Endo, T. and Yamamoto, T. (1978d). "Action of tin on the secretory effect of agents which stimulate gastric acid secretion rates." Toxicol. Appl. Pharmacol. 43: 417 - 421.

Yamaguchi, M., Kubo, Y. and Yamamoto, T. (1979). "Inhibitory effect of tin on intestinal calcium absorption in rats." Toxicol. Appl. Pharmacol. 47: 441 - 444.

Young, J. F., Gillard, R. D. and Wilkinson, G. (1964). "Complexes of Ruthenium, Rhodium, Iridium and Platinum with Tin (II) Chloride." J. Chem. Soc. 5176 - 5188.

Zalik, P. (1975). Outline of statistical Methods for Biological and Medical Research. Univ. Alberta Press. pp. 71 - 74.

Zedeck, M. J., Grab, D. J. and Sternberg, S. S. (1977). "Differences in the acute response of the various segments of rat intestine to treatment with intestinal carcinogen." Cancer Res. 37: 32 - 36.