CANADIAN THESES ON MICROFICHE

ISBN.

THESES CANADIENNES SUR MICROFICHE

National Library of Canada
Collections Development Branch

Canadian Theses on
Microfiche Service

Ottawa, Canada
K1A 0N4

Bibliotheque nationale du Canada
Direction du developpement des collections

Service des thèses canadiennes
sur microfiche

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

NL-339 (r. 82/08)

Canada

(57)

0-315-18221-0

National Library of Canada / Bibliothèque nationale du Canada

CANADIAN THESES ON MICROFICHE / *THÈSES CANADIENNES SUR MICROFICHE*

66130

NAME OF AUTHOR/*NOM DE L'AUTEUR* Patrick Ojeifo Uadia

TITLE OF THESIS/*TITRE DE LA THÈSE* The Uptake and Mode of Cytotoxic Action of Methotrexate - Immunoglobulin Conjugates

UNIVERSITY/*UNIVERSITÉ* Dalhousie

DEGREE FOR WHICH THESIS WAS PRESENTED/ *GRADE POUR LEQUEL CETTE THÈSE FUT PRÉSENTÉE* Ph.D.

YEAR THIS DEGREE CONFERRED/*ANNÉE D'OBTENTION DE CE DEGRÉ* 1984

NAME OF SUPERVISOR/*NOM DU DIRECTEUR DE THÈSE* Dr. A.H. Blair/Dr. T. Ghose

Permission is hereby granted to the NATIONAL LIBRARY OF CANADA to microfilm this thesis and to lend or sell copies of the film.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

L'autorisation est, par la présente, accordée à la BIBLIOTHÈQUE NATIONALE DU CANADA de microfilmer cette thèse et de prêter ou de vendre des exemplaires du film.

L'auteur se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans l'autorisation écrite de l'auteur.

DATED/*DATE* September 11, 1984 SIGNED/*SIGNÉ* [redacted]

PERMANENT ADDRESS/*RÉSIDENCE FIXÉ* Dept. of Biochemistry, University of Port Harcourt, Port, Harcourt, Rivers State, Nigeria

NL-91 (11-79)

THE UPTAKE AND MODE OF CYTOTOXIC ACTION OF METHOTREXATE-IMMUNOGLOBULIN CONJUGATES

by

© Patrick Ojeifo Uadia

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy at Dalhousie University

Department of Biochemistry,

Dalhousie University

Halifax, Nova Scotia

September, 1984

TABLE OF CONTENTS

		Page
	List of Figures	xi
	List of Tables	xiii
	Abstract	xiv
	Abbreviations	xvi
	Acknowledgements	xviii
	Introduction	1
A.	General	1
B.	Methotrexate: Structure	4
C.	Mechanism of action	7
(i)	The inhibition of DHFR	7
(ii)	Structure of DHFR	8
(iii)	The mode of interaction of MTX with DHFR and NADPH	11
(iv)	Kinetics of DHFR inhibition by MTX	15
(v)	The relationship between intracellular level of MTX and DHFR inhibition in living cells	17
(vi)	Implications of polyglutamate formation for MTX cytotoxicity	17
D.	Pharmacology of Methotrexate	19
(i)	Membrane transport	19

Page

(ii) Administration 22
(iii) Absorption 22
(iv) Distribution 22
(v) Metabolism 24
(vi) Excretion 25
(vii) Resistance 26
(viii) Toxicity 27
E. Immunoglobulin G 28
(i) Structure 28
(ii) The use of antibody in immunotherapy 32
(iii) Antibodies as carriers of cytotoxic agents in the treatment of cancer 34
F. Methods of coupling MTX to antibodies 35
(i) Diazotization 35
(ii) Mixed anhydride formation 38
(iii) Carbodiimides 39
(iv) The active ester method 41
G. Membrane transport of proteins and antibodies 42
(i) Endocytosis 42
(ii) Recycling of plasma membrane during endocytosis 46
H. Mechanisms of protein degradation in cultured cells 48

Page

I. Degradation of IgG by cells and cell extracts 52

J. Objectives 55

Materials and Methods 57

A. Materials 57

B. Mice, tumors and methods of transplantation 58

(i) EL4 lymphoma 58

(ii) Human melanoma M21 cell line 58

C Harvesting of tumors 59

(i) EL4 lymphoma 59

(ii) Human melanoma M21 cell line 59

D. Cell-counts and viability tests 60

E. Production of rabbit anti EL4 lymphoma antibody (AELG) 60

F. Production of rabbit antimelanoma M21 antibodies (AHMG) 61

G. Monoclonal antibody 225.28S to a high molecular weight melanoma associated antigen (MAB) 62

H. Fractionation of sera 63

I. Membrane immunofluorescence assay 63

J. Preparation of MTX-immunoglobulin conjugates 63

Page

K. Sodium dodecylsulfate polyacrylamide gel electrophoresis 64

L. In vitro uptake studies 64

(i) Uptake of MTX and its conjugates by EL4 cells 64

(ii) Efflux of free MTX from EL4 cells 65

(iii) Uptake of MTX and its conjugates by M21 cells 66

(iv) Efflux of MTX and catabolites of MTX-AHMG$_R$ conjugate from M21 cells 66

(v) Determination of cell-associated radioactivity 67

(vi) Protein determination 67

(vii) Calculation of endocytosis of conjugates 68

(viii) Catabolism of conjugates by M21 cells 68

(ix) Dihydrofolate reductase (DHFR) assay 69

(x) In vitro inhibition of growth of EL4 and M21 cells 69

M. In vivo uptake studies 70

(i) Uptake of MTX and its conjugates by tumor and normal tissues in EL4 lymphoma-bearing mice 70

(ii) Uptake of MTX and its conjugates by tumor and normal tissues in human melanoma M21-bearing

Page

nude mice 71
O. Preparation of homogenates of liver and EL4 cells 71
P. Determination and characterization of hydrolytic activity of liver homogenates on the MTX-NRG conjugate 71
Q Hydrolysis of the MTX-NRG conjugate by EL4 cell homogenates 72
R. DEAE-cellulose chromatography 72
S. Thin layer chromatography 73
Results 74
A. Properties of the MTX-antibody conjugates 74
(i) Molar incorporation and retention of drug activity 74
(ii) Retention of antibody activity 74
B. In vitro uptake studies with EL4 cells 77
(i) Uptake of free MTX 77
(ii) Uptake of MTX-NRG 77
(iii) Uptake of MTX-AELG 77
(iv) Efflux of MTX from EL4 cells preloaded with MTX 78

Page

C. in vivo uptake studies in EL4 lymphoma-bearing mice 78

(i) Tissue distribution of free MTX 85

(ii) Tissue distribution of MTX-AELG and MTX-NRG conjugates 85

D. Catabolism of MTX-NRG conjugate by EL4 cell homogenate 87

E. In vitro uptake studies with human melanoma M21 cells 87

(i) Uptake of free MTX 87

(ii) Uptake of MTX-NRG 94

(iii) Uptake of MTX-$AHMG_R$, MTX-$AHMG_{R+T}$ and MTX-MAB 94

(iv) Efflux of MTX from M21 cells preloaded with MTX 96

(v) Efflux of MTX-containing fractions from M21 cells preloaded with MTX-$AHMG_R$ 96

F. In vivo uptake studies in human melanoma M21-bearing nude mice 96

(i) Tissue distribution of free MTX 96

(ii) Tissue distribution of MTX-$AHMG_{R+T}$ and

Page

MTX-NRG 107

G. Catabolism of MTX-AHMG$_R$ by M21 cells 109

H. Determination and characterization of the hydrolytic activity of liver homogenates on the MTX-NRG conjugate 120

I. In vitro inhibition of growth of EL4 and M21 cells 121

Discussion 136

Conclusion 163

References 166

LIST OF FIGURES

Figure		Page
1.	Structural formulas of folic acid, aminopterin and methotrexate	6
2.	Interconversion of THF derivatives in relation to the generation and utilization of one-carbon units and the regeneration of THF from DHF	10
3.	The domain structure of human IgG1 (κ)	31
4.	Purity of IgG as determined by agar gel immunoelectrophoresis	76
5.	Uptake of free [^{3}H]MTX by EL4 lymphoma cells	80
6.	Uptake of MTX at various concentrations by EL4 lymphoma cells	82
7.	Uptake of [^{3}H]MTX-AELG and [^{3}H]MTX-NRG by EL4 lymphoma cells	84
8.	Uptake and clearance of free MTX, MTX-AELG and MTX-NRG in ascites fluid, serum and tissues of EL4-lymphoma-bearing mice	89
9.	Uptake of free MTX, MTX-AELG and MTX-NRG by tumor cells in EL4-lymphoma-bearing mice	91
10.	Bio-gel P100 elution profile of [^{3}H]MTX-NRG before and after incubation with EL4 lymphoma homogenate	93

Figure | Page

11. Uptake of free MTX by M21 cells 98
12. Uptake of conjugated MTX by M21 cells 100
13. Net uptake of MTX-MAB, MTX-AHMG$_R$ and MTX-AHMG$_{R+T}$ 102
14. Inhibition of the uptake of MTX-AHMG$_R$ by AHMG$_R$ or MTX 104
15. Efflux from M21 cells preloaded with MTX-AHMG$_R$ 106
16. Uptake and clearance of free MTX, MTX-AHMG$_{R+T}$ and MTX-NRG in serum and tissues of M21 melanoma-bearing nude mice 111
17. Uptake of free MTX, MTX-AHMG$_{R+T}$ and MTX-NRG by M21 melanoma-bearing nude mice 113
18. Gel chromatography of the efflux medium and cell homogenate after loading M21 cells with [^{3}H]MTX-AHMG$_R$ ... 115
19. DEAE-cellulose chromatography of low-molecular weight fraction from efflux medium 117
20. Breakdown of MTX-NRG conjugate after incubation with liver homogenates 125
21. Effects of antipain and N-α-p-tosyl-L-lysine chloromethyl ketone on hydrolysis of conjugate by mouse liver homogenates 127
22. DEAE-cellulose chromatography of low molecular weight fraction from MTX-NRG incubated with liver homogenates .. 133

LIST OF TABLES

Table		Page
1	Interactions between DHFR and MTX for the E. coli binary and L. casei ternary complexes	14
2.	DHFR inhibition by low molecular weight fragments in cell extract and efflux medium	119
3.	Residual radioactivity associated with M21 cells incubated with MTX-AHMG$_R$ in the presence of chloroquine or leupeptin	123
4.	Effect of several inhibitors and an activator of lysosomal enzymes on the breakdown of MTX-NRG conjugate by liver homogenate	129
5.	Influence of pH on the breakdown of MTX-NRG conjugate by liver homogenate	131
6.	In vitro inhibition of growth of EL4 and M21 cells by MTX, MTX-AELG, MTX-AHMG$_R$, MTX-NRG	135

ABSTRACT

The mode of the superior antitumor effectiveness of MTX-antitumor IgG conjugates has been investigated in murine EL4 lymphoma and human melanoma M21 cells by comparing the in vitro and in vivo uptake and level of accumulation of (a) free MTX (b) MTX linked to nonspecific IgG (NRG) and (c) MTX linked to IgG antibodies directed against antigens on the surface of cells. The rate of efflux of free or conjugated MTX from M21 cells in an environment depleted of drug as well as the ability of the cells to release free MTX or active MTX-containing fragments that can inhibit DHFR were also examined.

In the mouse EL4 model, more MTX was taken up by EL4 cells both in vitro and in vivo as the antitumor IgG conjugate than as the free drug or drug linked to NRG. The clearance from serum and tissues of MTX-IgG conjugates was slower than that of the free drug in EL4-bearing mice. In the human melanoma model in vitro, more MTX was taken up by the M21 cells as the antitumor IgG conjugate than as the free drug or drug linked to NRG, but in vivo, MTX-antiM21 IgG and MTX-NRG conjugates were taken up to the same extent. In vivo and in vitro, efflux from tumor cells of IgG-linked MTX was much slower than that of free MTX. Drug-containing fragments could be detected intracellularly and in the efflux medium when M21 cells were preloaded with MTX-antiM21 IgG and reincubated in conjugate free medium. These drug-containing fragments could inhibit DHFR. Therefore, reasons for

the increase in cytotoxic potential of MTX-IgG conjugates in vivo would include higher uptake of the IgG-linked MTX followed by slow sustained catabolism so that there is prolonged maintenance of intracellular active derivatives at a level higher than that of DHFR*.

*Results of my studies on the uptake of the MTX-AELG conjugate by EL4 cells have been published in Cancer Immunol. Immunother., 1983, 16, 127. One paper on the tumor and tissue distribution of MTX-AELG in EL4 lymphoma-bearing mice has been accepted for publication in Cancer Research and another on uptake of MTX linked to poly and monoclonal antimelanoma antibodies by human melanoma M21 cell line has been accepted by JNCI. In addition, parts of this work have been presented at the New York Academy of Sciences conference on macromolecules as drugs and as carriers for biologically active materials, (March, 1984), the 75th annual meeting of the American Association for Cancer Research in Toronto (May, 1984) and the Canadian congress of laboratory medicine in Halifax (June, 1984).

ABBREVIATIONS

AELG - Rabbit antiEL4 IgG

AHMG - Rabbit antihuman melanoma IgG

$AHMG_R$ - Rabbit antihuman melanoma IgG absorbed with human red blood cells

$AHMG_{R+T}$ - Rabbit antihuman melanoma IgG absorbed with human red blood cells and tissues

BSA - Bovine serum albumin

Con A - Concanavalin A

DCC - Dicyclohexyl carbodiimide

DEAE-cellulose - Diethylaminoethyl cellulose

DHF - Dihydrofolic acid

DHFR - Dihydrofolate reductase; 5,6,7,8 - tetrahydrofolate: NADP oxidoreductase EC.1.5.1.3

DMF - Dimethyl formamide

DTT - Dithiotreitol

EDTA - Ethylenediaminetetraacetic acid

EGTA - Ethyleneglycol-bis-(β-aminoethylether)-N,N,N^1,N^1-tetraacetic acid

^3H - Tritium; radioisotope hydrogen 3

^{125}I - Radioisotope iodine 125

^{131}I - Radioisotope iodine 131

IgG - Immunoglobulin G

Km - Michaelis-Menten constant

MAA - Melanoma-Associated Antigen

MAB - Monoclonal antibody 225.28S

MTX - methotrexate

MTX-AELG - Methotrexate-antiEL_4 IgG conjugate

MTX-MAB - Methotrexate-monoclonal antibody conjugate

MW - Molecular Weight

NADP - Nicotinamide adenine dinucleotide-phosphate

NHS - N-hydroxy-succinimide

NMG - Normal Mouse globulin

NRG - Normal Rabbit globulin

PBS - Phosphate buffered saline pH 7.4 (0.01 M sodium phosphate containing 0.145 M sodium chloride)

R_f - Retardation factor

SDS-PAGE - Sodium dodecylsulfate polyacrylamide gel electrophoresis

TAA - Tumor-associated antigen

TCA - Trichloroacetic acid

THF - Tetrahydrofolic acid

TLC - Thin layer chromatography

Vmax - Maximum velocity

ACKNOWLEDGEMENTS

I wish to express my gratitude to Drs. Blair and Ghose for their encouragement and excellent advice. I am grateful to Pam, Molly, Helen, Damaso, Ed and Shashi for their help and continued friendship. I thank Dr. Peter Dolphin for allowing me to use his Oxymat.

I acknowledge with thanks the financial support from the University of Port Harcourt and from the Medical Research Council of Canada (Grant no. MT 6922). I am grateful to Ms. Kathy Whalen for her skillful typing of this thesis. Finally, I wish to express my indebtedness to Rachel, Boma and Oje for their love, understanding and patience during the course of this work.

INTRODUCTION

A. GENERAL

Among the currently available methods for the treatment of cancer, chemotherapy constitutes a major therapeutic approach, along or in combination with surgery, radiotherapy and, to a lesser extent, immunotherapy. Most cancer therapeutic agents now in use are not truly tumor specific, they have equal detrimental effects on normal cells, particularly the rapidly proliferating ones in the gastrointestinal tract, bone marrow etc. This nondiscriminating mode of action severely limits the dose that can be given, in most cases to a level that will not kill all tumor cells. The effectiveness of cancer therapeutic agents is evaluated in terms of the therapeutic index which is the ratio between the median toxic dose and the median tumoricidal dose. Increase in tumoricidal effect compared to systemic toxicity will therefore increase the therapeutic effectiveness of a given anticancer drug.

One possible approach for increasing the effectiveness of antitumor drugs would be to find methods of altering their distribution in the body so as to increase their local concentration at the tumor sites, while a lower systemic concentration is maintained. In this way, the selectivity of their toxicity for the tumor cells might be enhanced. To achieve this, specific macromolecules, such as, receptor specific glycoproteins, polypeptide hormones and antibodies have been used as carriers for various cytotoxic drugs. Nonspecific carriers, such as, DNA, liposomes, red

blood cell ghosts, lectins, synthetic polymers have also been used (Ghose and Blair, 1978). Although this latter group lack tumor specificity, anticancer drugs bound to most of them have been reported to have increased therapeutic efficacy which can be attributed to inhibition of drug catabolism, slow release of drug from the complex (i.e. a depot effect) or increased endocytosis of macromolecule-bound drugs by tumor cells.

Targeting through the use of specific carriers is based on the assumption that the carrier will recognize and bind selectively to a receptor accessible to the circulating drug-carrier complex. Antibodies, by virtue of their unique specificity and high affinity for the antigen, are particularly attractive as selective carriers of toxic agents. A cytotoxic agent-antibody conjugate once transported to the cell surface or its vicinity may exert its cytotoxic effect in several ways. If the target site of the drug is on the cell surface then the increased concentration of the linked drug at the cell surface will result in high concentration of the active agent and produce cell kill. If the site of action is intracellular, for the carrier-linked drug to be effective, either the cell surface bound conjugate has to be internalized via the receptor for the carrier or the drug has to be dissociated and internalized by the receptor for the drug. For instance, a number of toxic proteins owe their extreme potency to the fact that they possess an enzymatically active A-chain which exerts the toxic effect by inhibiting protein synthesis and a B-chain which mediates the entry of the toxins into cells by binding

to cell-surface receptors. Since receptors for the B-chain of these toxins are present on the surface of most cells of sensitive animals, the unmodified toxins show little selective toxicity. This problem has been circumvented by conjugating antibodies to the enzymatically active A-chain moiety. Gilliland and Collier (1980) covalently attached the A-chain of Diphtheria toxin to antibody against Con A by means of a disulfide containing cross-bridge. This conjugate was toxic for 3T3 cells containing Con A on their surface but was not toxic in the same concentration range for cells lacking Con A on their surface.. Also, conjugates of A-chain of Diphtheria toxin and NRG were not toxic for cells coated with Con A, thus indicating that the A-chain antibody conjugate manifests its toxic activity towards its target cell through binding of the antibody moiety to its cell-surface antigen. It is assumed that after internalization, the A-chain is cleaved from the conjugate by intracellular proteolytic enzymes for it to exert its toxic effect (Edwards, 1983; Olsnes and Pihl, 1982). This has not been proved.

Chlorambucil, which exerts its cytotoxic effect by alkylating nuclear DNA, has been covalently coupled to antitumor antibodies. Chlorambucil-antitumor-antibody conjugates are much more effective tumor inhibitors (both in vivo and in vitro) than chlorambucil, antibodies alone or comparable amounts of chlorambucil bound to "normal" globulins (Ghose and Nigam, 1972, Ghose et al., 1972). The increased tumor inhibition by chlorambucil-bound antitumor antibody compared to that of equivalent amount of chlorambucil has been

explained, at least in part, by facilitation by the antitumor antibody moiety of the transport of chlorambucil across the cell membrane by endocytosis following capping (Guclu et al., 1975). It has not been determined if it is the intact conjugate or chlorambucil released from it by intracellular proteolytic enzymes that alkylates nuclear DNA.

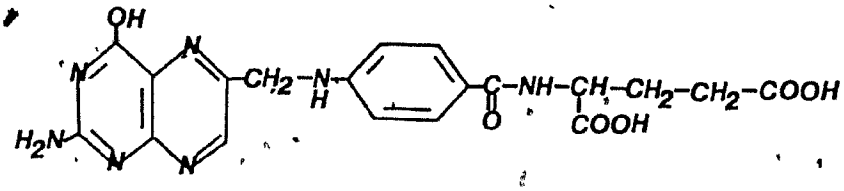
In this thesis, I have investigated the basis of the observed superior tumor inhibitory effect of the antimetabolite, MTX linked to a carrier antibody.

B. METHOTREXATE: STRUCTURE

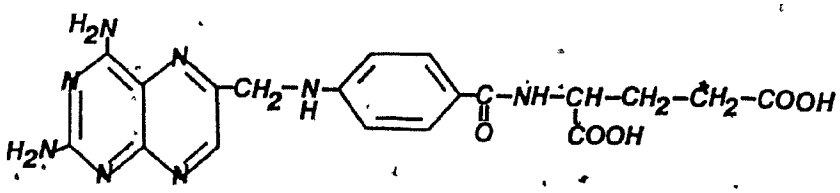
Since their clinical introduction in 1948, antifolates have become widely used as chemotherapeutic agents (Farber et al., 1948). Methotrexate, the most widely used antifolate in cancer chemotherapy, has an extensive role in the treatment of such diverse diseases as acute lymphocytic leukemia, non-Hodgkins lymphoma, osteosarcoma, choriocarcinoma, head and neck cancer, and breast cancer (Chabner, 1982). It has also become an important therapeutic alternative in the treatment of severe psoriasis (Weinstein, 1977) and in the suppression of graft-versus-host disease after bone-marrow transplantation (Blume, et al., 1980), as well as in the experimental treatment of various rheumatic diseases after primary therapy has failed (Willkens and Watson, 1982).

Methotrexate (amethopterin, 4-amino-4-deoxy-N^{10}-methyl-pteroylglutamic acid) differs in molecular structure from folic acid in that folic acid has a hydroxyl group in place of the 4-amino group on the pteridine ring and there is no methyl group at

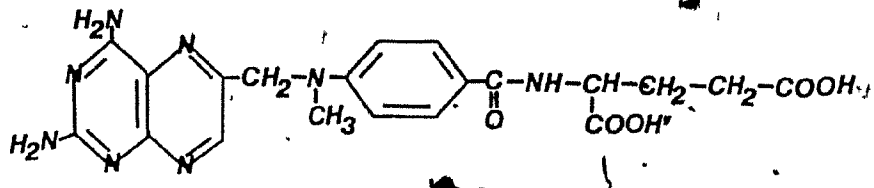
Figure 1: STRUCTURAL FORMULAS OF FOLIC ACID, AMINOPTERIN AND METHOTREXATE



FOLIC ACID



AMINOPTERIN



METHOTREXATE

the N^{10} position; it also differs from aminopterin in that the latter is not methylated at the N^{10} position (Figure 1). It is a dicarboxylic weak acid with pk_a values in the range of 4.8 to 5.5 (Liegler et al., 1969); hence, it is essentially ionized and lipid insoluble at physiological pH.

C. MECHANISM OF ACTION

(1) THE INHIBITION OF DHFR

MTX binds tightly to and inhibits dihydrofolate reductase (tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3, DHFR), the enzyme responsible for the conversion of folic acid to reduced folate cofactors (Bertino, 1975; Schornagel and McVie, 1983; Jolivet et al., 1983). DHFR catalyzes the NADPH-dependent reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF) (Figure 2). THF is a coenzyme in a number of one-carbon transfer reactions such as in the biosynthesis of serine, methionine, histidine, purines and thymidylate (Huennekens, 1968; Blakely, 1969; Radar and Huennekens, 1973). Since these metabolites in turn serve as precursors of proteins and nucleic acids, it is evident that their synthesis is obligatory for cell replication.

From the pharmacological viewpoint the most important function of THF is in the reaction whereby deoxyuridylate (dUMP) is converted to thymidylate (dTMP) by thymidylate synthetase (EC 2.1.1.45). The reaction producing thymidylate is unique among those involving the THF coenzymes because THF is used not only as a source of a one-carbon fragment but also as a reductant and hence is used in substrate rather

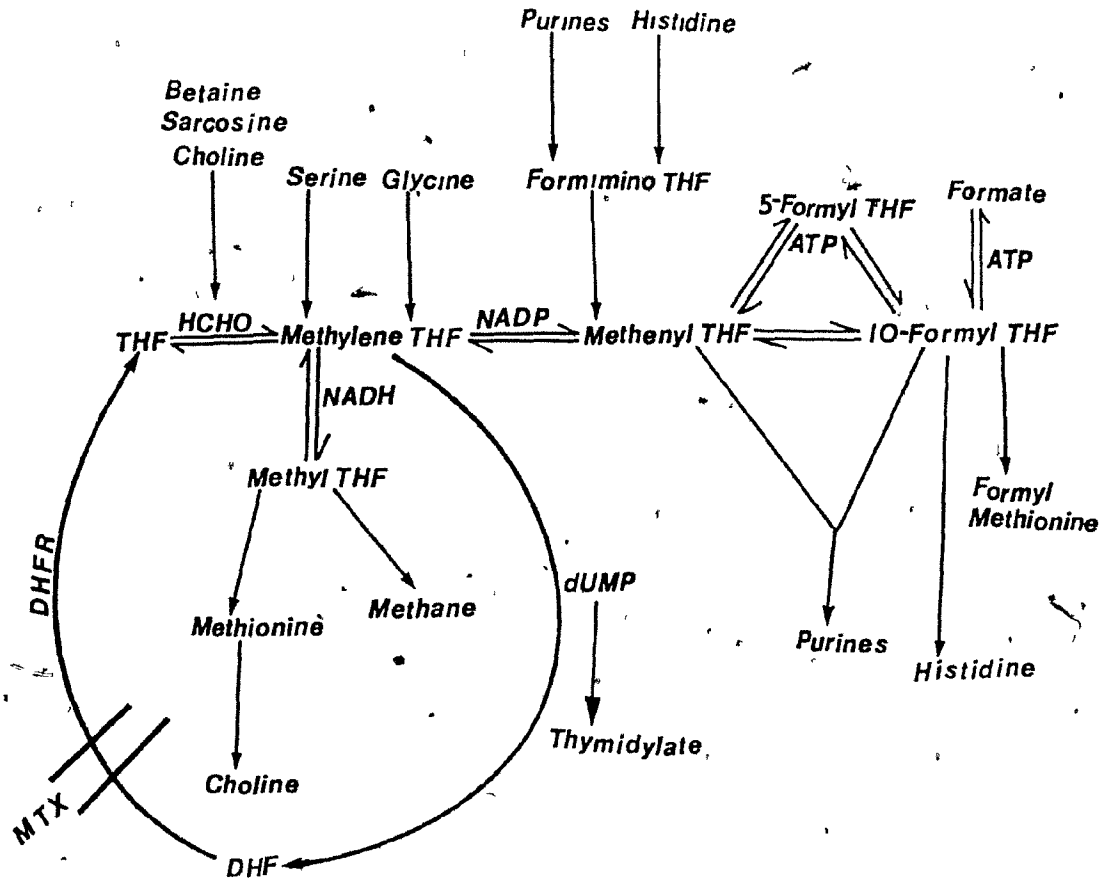
than coenzyme amounts with the formation of DHF. THF must be regenerated via the DHFR-catalyzed reaction in order to maintain the cellular pool of THF derivatives. Inhibition, then, of DHFR by MTX leads to a deficiency of thymidylate and thus to disruption of nucleic acid synthesis. This mechanism for interfering with DNA synthesis is thought to provide the biochemical basis for the cytotoxic action of MTX (Blakely, 1969).

(ii) STRUCTURE OF DHFR

The amino acid sequence of DHFR has been determined after purification of the enzyme from the following sources: an MTX-resistant strain of Escherichia coli, MB1428, [159 residues, MW 17,958 daltons (Bennett, et al., 1978)], an MTX-resistant strain of Lactobacillus casei [162 residues, MW 18,322 daltons (Bitar, et al., 1977)]; an MTX resistant strain of Streptococcus faecium, [167 residues, MW 20,800 daltons (Gleisner et al., 1974; Peterson, et al., 1975a)] and MTX-resistant line of mouse lymphoma L1210 cells [186 residues, MW 21,458 daltons (Stone et al., 1979)]. In addition, the structure has been determined by X-ray crystallography at 2.5 A resolution for the binary complex of MTX and DHFR from MTX-resistant E. coli (Matthews et al., 1977) and for the ternary complex of MTX, NADPH and DHFR from MTX-resistant L. casei (Matthews et al., 1978).

These studies have defined the overall shape of the enzyme molecule, identified regions of secondary structure and binding areas for inhibitor and cofactor. The polypeptide backbone is folded into an eight-stranded β-sheet with seven parallel strands and one antiparallel strand. The sheet begins at the NH_2-terminus and ends

Figure 2: Interconversion of THF derivatives in relation to the generation and utilization of one-carbon units and the regeneration of THF from DHF. Reactions leading to provision of 'active' one-carbon fragments are depicted in the top part and utilization of these 'activated' fragments in biosynthetic reactions is depicted in the lower part. Interconversion of THF and its various derivatives is represented along the centre line.


Purines
Histidine
Betaine
Sarcosine
Choline
Serine
Glycine
Formimino THF
5-Formyl THF
ATP
Formate
ATP
THF
HCHO
Methylene THF
NADP
Methenyl THF
10-Formyl THF
NADH
Methyl THF
Formyl Methionine
DHFR
Methionine
Methane
dUMP
Purines
Histidine
Thymidylate
Choline
MTX
DHF

with a single antiparallel strand at the COOH-end. About 30% of the backbone is involved in the β-sheet structure, the remaining secondary structure consisting of several helices and interconnecting loops.

(iii) THE MODE OF INTERACTION OF MTX WITH DHFR AND NADPH

It was observed that MTX binds to DHFR 10,000 to 50,000 times (6-8 kcal/mole) more tightly than does DHF (Blakely, 1969). The earliest attempts to explain this difference in binding between DHF and MTX noted the marked increase in basicity which accompanies the replacement of the 4-keto group of DHF by the 4-amino group of MTX and proposed that a higher degree of protonation of MTX (when bound) might lead to a tighter binding as the result of an extra ionic interaction (Baker, 1959). This proposal prompted most of the activity aimed at discovering preferred protonation sites and led to the determination of the ternary complex of MTX-NADPH-DHFR by X-ray crystallography.

The X-ray crystallography experiments of Matthews et al., (1978) revealed that MTX is bound in a cavity which is 15A° deep and cuts across one whole face of the enzyme. It is bound in an open conformation with its pteridine ring nearly perpendicular to the aromatic ring of the p-aminobenzoyl group: the overall conformation is similar in both the binary and ternary complexes. The pyrimidine end of the pteridine ring is buried in a primarily hydrophobic pocket. Most of the face of the pyrazine ring is completely exposed to solvent in the binary complex but covered by NADPH in the ternary complex in a manner that would allow reduction of DHF at N(5)-C(6) as required in an active-enzyme-substrate complex (Huennekens and Scrimgeour, 1964).

One side of the cavity provides a binding site for the nicotinamide ring of NADPH while the remainder of the coenzyme molecule fits in a shallow groove. More than half the MTX and coenzyme interactions are with the first 50 residues of the enzyme, many of which are strongly conserved in the various species that have been examined. Table 1 summarizes some information about interactions between specific enzyme residues and MTX in both the binary and ternary complexes.

So far, there has not been unanimous agreement on a preferred protonation site, to account for the tighter binding of MTX to DHFR. The N_1 of the pteridine ring of the bound MTX has been proposed as a site that would be more easily protonated whereas it is not so in bound DHF. Baker and Ho (1964) suggested that the proton was donated by an enzyme histidine residue; however, the recent amino acid sequence studies have not revealed a totally conserved histidine and the X-ray studies failed to show a histidine residue anywhere near the pteridine binding pocket in either L. casei or E. coli DHFR (Matthews et al., 1978). Matthews et al., (1978) note that the only potential proton donors in the neighbourhood of the pteridine ring (in the enzyme-inhibitor complexes) are Asp-29 and Thr-136; although the current sequence results still indicate Thr-136 as totally conserved, Asp-29 is known to be Asn-29 in L1210 and some other mammalian DHFR. However, Hood and Roberts (1978) have shown that only about one-third of the difference in binding energy between DHF and MTX could be attributed to protonation, which accords with several spectroscopic studies indicating that as well as the protonation difference, the two

Table 1: INTERACTIONS BETWEEN DHFR AND MTX FOR THE E. COLI BINARY AND L. CASEI TERNARY COMPLEXES.

Table 1

INTERACTIONS BETWEEN DHFR AND MTX FOR THE E. COLI BINARY AND L. CASEI TERNARY COMPLEXES

MTX CONSTITUENT	E. COLI	L. CASEI	TYPE OF INTERACTION COMMENTS
Pteridine ring	Ile-4	Leu-4	Hydrophobic
	carbonyl of Ile-4 Peptide 5-6	Carbonyl of Leu4 Peptide 5-6	H-bond to 4-NH_2
			Hydrophobic or pi-pi, approaches N_1, C_2, 2-NH_2 and N_3
	Ala-6	Ala-6	Hydrophobic
		Leu-19	Hydrophobic, pyrazine ring
	Asp-26	Asp-26	Charge interaction and H-bonds; approaches N_1, 2-NH_2 and N_8
	Leu-27	Leu-27	Hydrophobic
	Phe-30	Phe-30	Hydrophobic
	Ile-96	Carbonyl of Ala-97	van der Waals/hydrophobic
	Thr-116	Thr-116	H-bond to 2-NH_2
		Nicotinamide-ring of NADPH	hydrophobic
N_{10} Methyl	Ser-48	Ser-48	Hydrophobic
p-amino	Leu-28	Leu-28	Hydrophobic
benzoyl	Ile-49	Phe-49	Hydrophobic
	Leu-54	Leu-54	Hydrophobic
Glutamate	Lys-31 or H_2O	His-28	Hydrophobic
	Arg-57	Arg-57	Charge interaction and α-carboxyl H-bond

molecules are bound somewhat differently. This has been confirmed by Charlton and Young (1979) who showed that the pteridine ring of DHF is bound upside down compared to MTX.

(iv) KINETICS OF DHFR INHIBITION BY MTX

The inhibitory effect of MTX on the chicken liver DHFR was discovered independently by Futterman (1957), by Osborn et al., (1958), and by Zakrzewski and Nichol (1958). These studies established that very low concentrations of MTX (10^{-8}M to 10^{-9}M) produce marked inhibition of the chicken liver DHFR and when the data were treated according to the method of Lineweaver and Burk (1934) the results apparently indicated that the inhibition was non-competitive with respect to DHF (Osborn et al., 1958). These assays were performed at pH 7.5.

In contrast, Werkheiser (1961) reported that, in the combination of MTX with rat liver DHFR at pH 6.1, equilibrium is so far in favour of the complex that when the enzyme activity is titrated by MTX there is almost a straight line relationship between residual enzyme activity and the amount of inhibitor added. Conversely, when activity was studied as a function of amount of enzyme added to a constant amount of MTX, a line parallel to the control and intercepting the x-axis was obtained. Behaviour of this type is characteristic of enzyme-inhibitor complexes with apparent dissociation constants so low that they are small even compared with the enzyme concentration and has been termed 'stoichiometric inhibition' (Werkheiser, 1961). At concentrations of a stoichiometric inhibitor inadequate to cause

complete inhibition of enzyme, practically all the inhibitor is enzyme-bound. Since the classical kinetic treatment assumes that all inhibitor is free, the equations used by Lineweaver and Burk in the graphical treatment of kinetic data are not appropriate. The presence of such a stoichiometric inhibitor increases both slope and intercept of a double reciprocal plot (1/v versus 1/[s]) in the same manner as a classical non-competitive inhibitor. A double reciprocal plot therefore cannot distinguish between classical non-competitive inhibition and stoichiometric inhibition.

At pH 7.5, highly purified chicken liver DHFR gave kinetic data that were inconsistent with stoichiometric inhibition (Matthews and Huennekens, 1963). The complex formed by this preparation of DHFR with MTX was demonstrated to be readily dissociated during chromatography on hydroxylapatite at pH 6.5 enabling elution of the enzyme and inhibitor in separate fractions. A similar separation of the components of the complex was obtained by chromatography on DEAE-cellulose. These studies demonstrate that, at lower pH, MTX acts as a "pseudoirreversible" inhibitor of DHFR whereas at higher pH it behaves as a reversible inhibitor; indeed, with purified DHFR obtained from Ehrlich ascites cells, Bertino et al., (1964) showed that it exhibited two pH optima with DHF as substrate: pH 5.9 and pH 7.5. Tighter binding and greater inhibition were produced by MTX at pH 5.9 than at pH 7.6 and at the latter pH, the inhibition was shown to be competitive. This pattern of MTX inhibition of DHFR has tremendous implications for its role as a cytotoxic agent in vivo as shown below.

(v) THE RELATIONSHIP BETWEEN INTRACELLULAR LEVEL OF MTX AND DHFR INHIBITION IN LIVING CELLS

Jackson and Harrap (1973) found that 95% of DHFR activity could be inhibited before the cellular growth rate was appreciably reduced. Only small amounts of DHFR appeared to be sufficient to maintain THF pools. Goldman (1974) showed that the presence of free (unbound) intracellular MTX was critical for the inhibition of DNA synthesis. When the free drug was removed, DNA synthesis was rapidly resumed. The explanation for this phenomenon is that only a small fraction of DHFR activity is required to sustain THF synthesis and that the intracellular DHF level can rise over several orders of magnitude when DHFR is inhibited by MTX. DHF, when present in the cell at low levels, cannot compete with MTX for its target enzyme DHFR, however, functionally important competition does occur when the DHF level is high, thereby diminishing the drug's effectiveness (Goldman, 1975; Goldman, 1977). Thus, for optimal cytotoxicity the intracellular concentration of MTX has to exceed the binding capacity of DHFR.

(vi) IMPLICATIONS OF POLYGLUTAMATE FORMATION FOR MTX CYTOTOXICITY

Like folates, MTX is extensively metabolized intracellularly to polyglutamate derivatives. Baugh et al., (1973) first observed these derivatives in red cells and other investigators subsequently found them in the livers of patients who had received MTX (Jacobs et al., 1977a). Such compounds have now been identified in various murine and human tissues. (Whitehead et al., 1975; Whitehead 1977; Rosenblatt et al., 1978a, Poser et al., 1980; Poser et al., 1981; Witte et al.,

1980; Gewirtz et al., 1980; Galivan 1980; Fry et al., 1982; Krakower et al., 1982; Schilsky et al., 1980; Jolivet et al., 1982a; Jolivet et al., 1982b). The formation of MTX polyglutamates allows the accumulation of intracellular drug far above the levels of the parent compound that would otherwise exist in equilibrium with extracellular drug. These compounds have at least equal affinity for DHFR (Jacobs et al., 1975; Clendeninn et al., 1983) but, in intact cells, the methotrexate polyglutamates appear to dissociate from dihydrofolate reductase at a slower rate than methotrexate, indicating that they are potentially less reversible inhibitors than the parent drug.

The most striking property of the polyglutamates is their ability to remain within the cell in the absence of extracellular drug (Fry et al., 1982; Jolivet et al., 1982b; Jolivet and Chabner 1983; Rosenblatt et al., 1978b; Balinska et al., 1981), in contrast to the parent compound, which rapidly leaves the cells after extracellular drug disappears. Retention is clearly influenced by chain length; derivatives that contain 3 or 4 additional glutamates are retained for up to 24 hr in the absence of external drug (Jolivet and Chabner, 1983) whereascompounds with a shorter chain length have proportionately shorter retention times. Longer retention is associated with prolonged inhibition of DHFR and extended cytotoxicity (Jolivet et al., 1982b).

In addition to inhibiting DHFR, methotrexate polyglutamates inhibit other folate requiring enzymes not affected directly by methotrexate itself. It is well established that folate polyglutamates have a much greater affinity for folate-requiring

enzymes than do the corresponding monoglutamate derivatives (McGuireet al., 1980). Preliminary experiments have shown that the addition of one glutamyl residue to methotrexate transforms the drug into a potent direct inhibitor of both thymidylate synthetase (Szeto et al., 1979) and aminoimidazolecarboxamide ribonucleotide transformylase (Baggott, 1983); the latter is one of the enzymes involved in de novo purine synthesis.

D. PHARMACOLOGY OF METHOTREXATE

(1) MEMBRANE TRANSPORT

Studies of MTX transport in vitro in mammalian cells date from 1962 when Fischer suggested that uptake of MTX in the L5178Y mouse leukemia cell represented a mediated process rather than simple diffusion (Fischer, 1962). Kessel et al., (1965) demonstrated a correlation between the membrane transport of MTX and cytotoxicity toward tumor cells when they showed that the percent increase in survival of mice bearing a variety of murine leukemias in the ascitic compartment was directly proportional to the uptake of MTX into the tumor cell in vitro. It has now been established that MTX transport in several mammalian cells is mediated by a high-affinity carrier (Goldman, 1971). MTX shares this carrier transport system with 5-methyltetrahydrofolate and 5-forymltetrahydrofolate (citrovorum factor, leucovorin, folinic acid). Influx of MTX is saturable with a Km (MTX concentration at which the rate of influx is 50% of maximal) of about 5 μM and it is pH and temperature sensitive. Folic acid, when present in high concentrations (10 μM) can utilize this same

route to a small extent, but generally enters the cell by another process, as suggested by the observation that sulfhydryl inhibitors abolish MTX influx but have little effect on folic acid uptake (Goldman, 1971; Radar et al., 1974). At high extracellular MTX concentrations (in excess of 20 μM), a second drug entry mechanism comes into play in addition to the high-affinity carrier (Warren et al., 1978; Hill et al., 1979). This poorly characterized process which probably involves diffusion is less efficient than the high affinity carrier but it accounts for the major fraction of drug that enters cells at high concentrations and explains the ability of "transport-resistant" cells to take up MTX at high extracellular concentrations.

MTX is transported into the cell by an active process, accumulating within the cell to levels of exchangeable intracellular MTX (the free drug in excess of the tightly bound fraction) exceeding the extracellular MTX concentration (Bender 1979; Goldman et al., 1968). Based on the observation that vincristine and some metabolic inhibitors such as azide, 2,4-dinitrophenol and dicoumarol can augment net influx of MTX (Bender, 1979; Fry et al., 1980; Fyfe and Goldman, 1973; Goldman, 1971; Hill et al., 1979; Henderson and Zevely 1980; Goldman, 1969; Radar, et al., 1974) whereas glucose and pyruvate decrease net influx (Henderson and Zevely, 1980; Goldman, 1969), Goldman has suggested an ATP-dependent system for pumping MTX out of the cell implying that influx and efflux are mediated by the same carrier (Goldman, 1971). This model proposes that the driving force

for MTX influx is due to a gradient of organophosphates between the intracellular and extracellular compartments. However, following the demonstration that various ions also decrease influx of MTX, Henderson and Zevely have proposed that the actual driving force for MTX influx is due to a gradient of inorganic phosphate between the intracellular and extracellular compartments (Henderson and Zevely, 1980; Henderson and Zevely, 1982).

Based on a mathematical evaluation of influx and efflux kinetics of MTX in isolated L1210 cells, Dembo and Sirotnak (1976) proposed that there are two distinct carrier systems involved in the mediation of MTX influx and efflux. This two carrier model has been supported by the observations that:

i. It is possible to select for MTX-resistant L1210 cells that demonstrate a sevenfold reduction in V_{max} for influx of MTX without any detectable change in the rate constant for efflux (Sirotnak et al., 1982; Sirotnak et al., 1981a).

ii. It is possible to inhibit irreversibly and completely block influx of MTX by means of carbodiimide-activated substrates without producing similar blockage of efflux (Henderson et al., 1980). The opposite kind of effect, in which efflux of MTX is greatly inhibited but influx is only slightly inhibited, has been obtained by treating cells with low concentrations of probenecid (Sirotnak et al., 1981).

While Dembo et al., (1984) have proposed essentially a single ATP-dependent efflux carrier, Henderson and Zevely (1984) have

proposed at least 3 components in MTX efflux. These efflux routes include the MTX influx carrier and two additional components which can be distinguished by their differential sensitivities to inhibition by bromosulfophthalein. These latter two components are induced by glucose and inhibited by azide and thus appear to be the same ATP-dependent efflux pump for MTX that has been proposed by Dembo et al., (1984) and which was originally described by Goldman (1971, 1969).

(ii) ADMINISTRATION

MTX can be administered orally, intramuscularly, intravenously, intra-arterially, intra-articularly, or intrathecally in a wide variety of dose levels and dosage regimens.

(iii) ABSORPTION

MTX is well absorbed from the gastrointestinal tract by active transport (Chungi, et al., 1978) at ~~low doses~~. At higher doses, absorption is incomplete; in this case, bioavailability may be enhanced by subdivision of the dose as opposed to a single large dose (Stuart, et al., 1979).

(iv) DISTRIBUTION

The first attempts to define the distribution and disposition of MTX in a comprehensive manner were reported by Zaharko et al., (1971) in their study of [^{3}H]MTX in mice. The primary elements of their model were:

i. Elimination of MTX by renal excretion.

ii. An active enterohepatic circulation.

iii. Metabolism of at least a small fraction of drug within the

gastrointestinal tract by intestinal flora and

iv. Multiple drug half-lives in plasma, the longest of which was found to be approximately three hours.

Each of these elements has been observed in man. The volume of distribution of MTX is approximately that of total body water. The drug is loosely bound to serum albumin with approximately 60% binding at or above 1 μM concentrations in plasma (Steele et al., 1979a). It can be displaced from this binding by weak organic acids such as aspirin (Leigler et al., 1969).

Organ distribution of MTX is directly correlated with the presence or absence of specific transport systems (Zaharko and Dedrick, 1977). MTX is rapidly transported into liver, kidney, skin and intestinal mucosa, whereas penetration into brain, fat and skeletal muscle appears to be poor (Anderson et al., 1970; Comaish and Juhlin, 1969). Extremely high MTX levels have been found in gall bladder bile, suggesting that biliary recirculation of the drug is quantitatively important. Bile may contain up to 20% of the administered dose (Calvert et al., 1977). Distribution of MTX into interstitial fluid spaces such as the cerebro-spinal fluid (CSF), pleural and peritoneal cavities occurs slowly by passive transport (Dedrick et al., 1975). These "third spaces", especially when pathologically increased as in ascites or pleural effusion may act as reservoirs from which MTX is slowly released back to the plasma, thus prolonging the time of exposure to toxic levels and resulting in increased toxicity (Evans and Pratt, 1978; Wann et al., 1974).

(V) METABOLISM

Although there is evidence that MTX is metabolized by microorganisms in the large intestine (Valerino et al., 1972), significant metabolism of MTX was only reported in 1976 by Jacobs et al., (1976), who identified 7-hydroxymethotrexate (7-OH-MTX) in the urine of patients receiving high-dose MTX. Lankelma et al., (1980; 1978) and Jacobs et al., (1977b) have demonstrated that conversion of MTX to 7-OH-MTX increased with subsequent infusions which suggests induction of a converting enzyme, probably aldehyde oxidase which is detectable in human liver tissue. 7-OH-MTX is an ineffective inhibitor of DHFR, but as it is four times less soluble than MTX it may contribute to the renal failure associated with high dose MTX treatment (Jacobs, et al., 1977b). In vitro, 7-OH-MTX has also been shown to decrease the amount of exchangeable intracellular MTX (Lankelma et al., 1980; Gaukroger et al., 1983; Fabre et al., 1984) suggesting that it interferes with rather than adds to MTX cytotoxicity.

A second metabolite is 2,4-diamino-N^{10}-methylpteroic acid (DAMPA), which has been shown to interfere with MTX radioimmunoassay (Donehower, et al., 1979). DAMPA is believed to be formed during enterohepatic circulation of MTX, in which glutamate is cleaved from the MTX molecule by intestinal bacterial carboxypeptidase. Like 7-OH-MTX, DAMPA has limited solubility in water and it is a weak inhibitor of DHFR.

Much more important metabolites of MTX are the poly-γ-glutamyl

derivatives described above which have now been found in a variety of normal and neoplastic human and animal tissues (Galivan, 1980; Gewirtz et al., 1979; Jacobs, et al., 1977a; Rosenblatt et al., 1978b; Schilsky, et al., 1980; Whitehead, 1977). This third type of metabolite which is formed by the addition of one or more glutamyl residues at the γ-position of MTX, may persist in human liver for months after drug administration (Jacobs et al., 1977a). Unlike 7-OH-MTX and DAMPA, the polyglutamates of MTX are potent inhibitors of DHFR and cell replication (Galivan, 1980; Rosenblatt et al., 1978b; Shilsky et al., 1980). The formation of polyglutamates has been associated with prolonged inhibition of DNA synthesis in cultured human fibroblasts, even after removal of exchangeable MTX (Gewirtz et al., 1979; Rosenblatt et al., 1978b) suggesting either tight intracellular binding to DHFR or decreased efflux. Polyglutamate synthesis increases as the dose of MTX is increased (Rosenblatt et al., 1978b). In the presence of folinic acid, a marked reduction in MTX polyglutamation in vitro was found (Rosenblatt et al., 1981). It has not been determined whether this resulted from inhibition of MTX uptake by the cell or from competitive inhibition of polyglutamate formation. Poser et al., (1981) have investigated polyglutamate formation in mouse small intestine as well as in a variety of murine tumors in vivo and found that polyglutamate synthesis was greater in all of the tumors studied than in the small intestine.

(vi) EXCRETION

MTX is predominantly eliminated via renal excretion of unchanged

drug. At very low plasma concentrations, MTX appears to be reabsorbed by the kidney (Huffman et al., 1973). At higher concentrations, the renal clearance of MTX is relatively constant (Huffman, et al., 1973) and exceeds that of inulin clearance (Leigler et al., 1969), indicating that the antifolate is not only filtered but is also actively secreted by renal tubular cells. Fecal excretion of i.v. administered MTX does not generally exceed 1 to 2% of the administered dose, however, fecal excretion after oral administration is proportional to dosage due to limitations in intestinal absorption (Bleyer, 1978).

(vii) RESISTANCE

Resistance to MTX can be a natural characteristic of some tumor cells or it can be acquired by sensitive cells. Impaired membrane transport of MTX has been recognized since 1962 as a mechanism of natural resistance to MTX in vitro in a strain of L5178Y leukemia cells (Fischer, 1962). This has now been demonstrated in tumor cells exposed to MTX in vitro (Hill et al., 1979; Sirotnak et al., 1981b). Tumor cells of various mouse and human cell lines containing DHFR with decreased binding affinity for MTX have been shown to be resistant to the drug (Hanggi and Littlefield, 1974; Flintoff and Essani 1980; Haber et al., 1981; Melera et al., 1980; Goldie et al., 1980). In a group of five mouse leukemias not previously exposed to MTX, Jackson and Niethammer (1977) found a correlation between cytotoxicity and DHFRaffinity for MTX, that is, the lower the affinity for MTX, the less the cytotoxicity. As MTX can only lead to THF depletion when dUMP is converted to dTMP, a reduced activity or absence of

thymidylate synthetase would lead to drug resistance. This was suggested by Moran et al., (1979) and has recently been demonstrated in cultured mouse mammary tumor cells by Ayusawa et al., (1981).

Natural resistance to MTX has been demonstrated in cells with elevated intracellular levels of DHFR, such as human melanoma cells (Kufe et al., 1980) or in cells that have the capacity of accelerated synthesis of DHFR, like human acute myeloid leukemia cells (Bertino et al., 1977). Acquired resistance with elevated DHFR levels has now been shown to be the result of gene amplification (Harding et al., 1970; Schimke, 1980). This occurs when cells are exposed at first to low drug levels and surviving cells are then treated with gradually increasing concentrations of MTX. In this stepwise selection process, initially small extrachromosomal DNA elements are formed, called double minute chromosomes. These double minutes contain the amplified genes for DHFR, but they are associated with unstable drug resistance: these cells revert to normal DHFR levels in the absence of MTX. In cells with stable resistance the amplified genes have been demonstrated on the long arm of the number 2 chromosome (Schimke, 1980).

(viii) TOXICITY

Toxicity from MTX has been shown to be a function of both drug concentration and time of exposure (Chabner and Young, 1973; Pinedo and Chabner, 1977; Pinedo et al., 1977). The dose required to produce toxicity varies from organ to organ. There appears to be a critical concentration threshold that must be exceeded before organ toxicity

will occur (Chabner and Young, 1973). Likewise a critical time of exposure to suprathreshold levels must be maintained before toxicity occurs. For bone marrow and gastrointestinal epithelium the plasma concentration and time thresholds appear to be 2 x 10^{-8}M and about 42 hr respectively (Levitt et al., 1973; Young and Chabner, 1973). The most common side effects of MTX are myelosuppression, mucositis of the gastrointestinal tract and a transient hepatitis-like condition. Nausea, anorexia and vomiting also are reported side effects.

At high MTX plasma levels or in the presence of renal insufficiency, severe impairment of the renal function may develop. This has been associated with precipitation of the drug in renal tubules on account of insufficient hydration and acid urine (Pitman et al., 1975; Stoller et al., 1975). In urine MTX was found to be 10 times less soluble at pH 5.5 than at pH 7 (Pitman et al., 1975; Stoller et al., 1975). Renal damage may occur especially in the presence of high concentrations of 7-OH-MTX, which is less soluble than MTX and can compete with MTX for tubular cell transport.

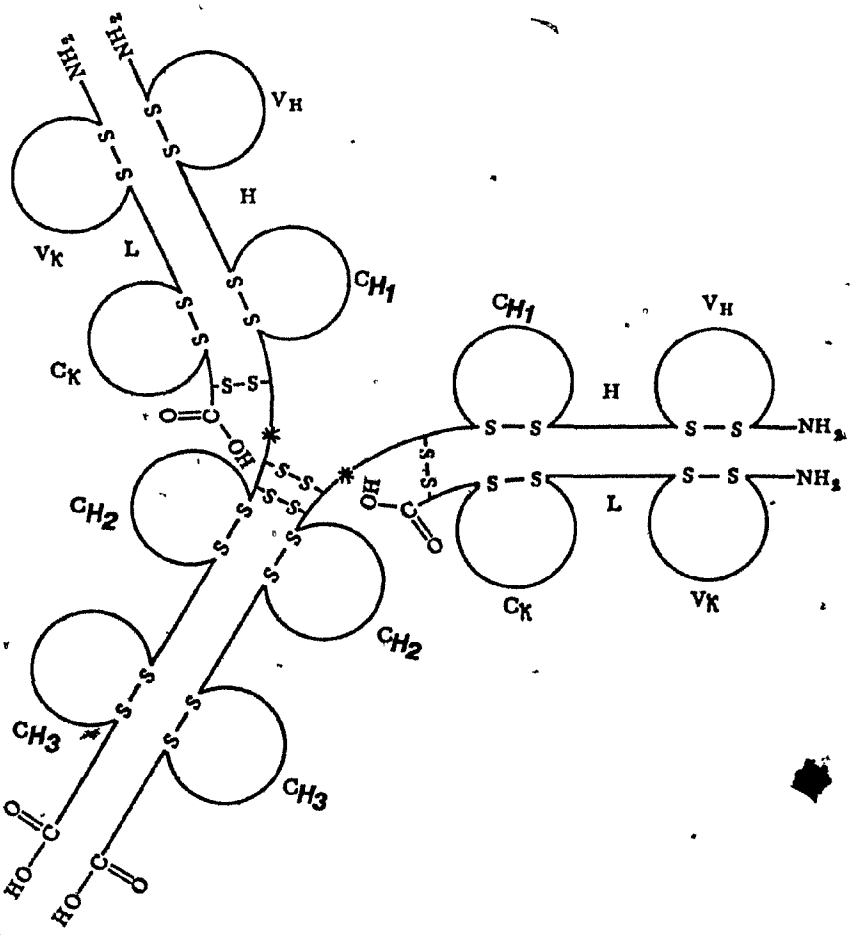
E. IMMUNOGLOBULIN G

(1) STRUCTURE

The IgG molecule (MW 150,000 daltons) has a symmetric structure consisting two heavy (H) chains (MW 53,000 daltons each) which are attached to one another by means of two or more disulfide bonds near the middle. Above the middle of each H chain (toward the aminoterminal ends) a light (L) chain is attached at a point close to its carboxyterminal end, by means of a disulfide bond (Figure 3). The

L chains have a MW of about 23,000 daltons (Edelman and Poulik, 1961) and are made up of two antigenically distinct types κ (kappa) and λ (lambda) (Deutsch et al., 1955; Burtin et al., 1956; Korngold and Lipari, 1956). Each IgG molecule consists of two rather well defined amino acid sequences designated the variable "V" region and the constant "C" region. The C region is that carboxyl terminal portion of the chain which has the same primary structure as in all other chains of the same class, subclass, and type; the V region is the amino terminal portion of the chain which can be made up of a great many different primary structures even within one subclass and type (Fleischman et al., 1962). Amino acid sequencing of immunoglobulins have shown that the polypeptide chains consist of a number of globular "domains" or "homology units", comprising 110 to 120 amino acids (Edelman, 1970). The domains are closed in a loop by a single disulfide bond. When detached from the intact immunoglobulin molecule, different domains do not tend to bind to each other except for pairs of homologous domains (e.g. C_K and C_{H1}), which interact strongly with one another. The complement-binding site is associated with CH_2 (Kehoe and Fougereau, 1969), as is the carbohydrate fraction of the IgG molecule (Hill et al., 1966). The sites that specifically bind to antigens are solely found in the V domains (Edelman 1973). The part of the IgG molecule that attaches to phagocytic cells is situated in the CH_3 domain (Yasmeen et al., 1976). Normally IgG is present in a closed conformation but electron microscope observations have shown that upon reacting with its antigen, both antibody-specific

Figure 3. The domain structure of human IgG1 (κ). The loops formed by the interchain disulfide bonds are globular units designated as domains. These are called V domains in the variable and C domains in the constant regions. IgG H chains have three C domains (C_{H1}, C_{H2} and C_{H3}). The L chains have one V and one C domain (V_κ and C_κ or Vλ and Cλ). The hinge regions in the H chains are indicated by asterisks. [From van Oss, C.J. Diagrammatic representation of the tetrapolypeptide IgGI molecule with light chains of the κ-type. In Greenwalt, T.J., and Steane, E.A. (eds): Blood Banking, vol II (CRC Handbook Series in Clinical Laboratory Science). (c) CRC Press, Inc., Cleveland, 1977.]


NH2
NH2
VH
H
VK
L
CH1
CK
S-S
O=C-OH
CH2
CH3
HO-C=O
CH1
VH
H
NH2
L
CK
VK

halves can fold out at the hinges as far as 180° (Valentine and Green, 1967).

Digestion of IgG with papain in the presence of cysteine splits the molecule into three fragments of about equal size with a MW of about 50,000 daltons. These consist of two Fab' fragments and one Fc fragment (Porter 1959). Digestion with pepsin yields $F(ab')_2$ fragment with a MW of about 106,000 daltons along with dialyzable polypeptides (Nisonoff et al., 1960). The $F(ab')_2$ fragment is composed of two identical moieties, each containing an antibody combining site and linked together by a disulfide bond.

(11) THE USE OF ANTIBODY IN IMMUNOTHERAPY

The suggestion by Ehrlich (1909) that there might be differences between normal and neoplastic cells that could evolve into vaccination programs against cancer, was subsequently supported by the finding that the rejection of tumor grafts in inbred strains of mice was based on immune reactions against tumor-specific antigens, (Gross, 1943; Foley, 1953). This led to an active interest in the use of antibodies for immunotherapy. The scientific rationale for immunotherapy is based on the assumption that most animal and human tumors express specific cell surface antigens (Klein, 1975). They are often recognized as foreign or "non-self" by the tumor-bearing host, and thus an immune response is mounted against these antigens. Despite the presence of 'foreign' antigens on the surface of tumor cells as targets in many types of human cancers, immunodeficiency is a common phenomenon that becomes more severe as the cancer progresses. In addition, the conventional therapeutic approaches to cancer, including

surgery, radiotherapy and chemotherapy have immunosuppressive effects that can persist (Alexander et al., 1967).

Based on the apparent failure of the host defence system and the correlation between prognosis and a good immune response, immunotherapy would have the following objectives.

i. Restoration of the patient's full immunologic capacity. Some of the methods include immunostimulation with microbial or synthetic immunostimulants, for example, living Bacillus Calmette-Guerin (BCG) and BCG extracts, Corynebacterium parvum and C. granulosum, lipopolysaccharides or gram-negative bacteria, pertussis vaccine, vaccinia virus, polysaccharide extracts of fungi. Another approach is removal from the plasma of circulating soluble tumor antigens or antigen-antibody complexes.

ii. Protection against or reversal of the immunosuppressive effects of radiation, chemotherapy, surgery, age and other factors.

iii. Induction, restoration or increase in specific tumor immunity. Active specific immunization can be carried out with unmodified tumor cells, modified tumor cells or tumor antigens. Passive transfer of immune serum and adoptive transfer of immunologically active cells e.g., lymphocytes can also be done. These immunopotentiators may also enable the recipient to respond to otherwise poorly immunogenic antigens.

iv. In addition to production of antibodies, immunotherapy hyperactivates the various components of general host defence which include the reticuloendothelial and macrophage activity.

Although these objectives have been met with various degrees of success in some clinical trials that have been carried out, they are not consistent (Gutterman and Hersch, 1982).

(iii) ANTIBODIES AS CARRIERS OF CYTOTOXIC AGENTS IN THE TREATMENT OF CANCER

It is a well known fact that chemotherapeutic agents are not selective in their action against cancer cells as they damage all proliferating cells including those of bone marrow, lymphoid tissue and gastrointestinal and genitourinary epithelium. Since the need to preserve vital tissues reduces the maximum potential dose of cytotoxic drugs, the possibility of developing methods to transport nondiscriminating toxic molecules selectively to target sites has attracted considerable attention (Rubens, 1974; Gregoriadis, 1977; Ghose and Blair, 1978).

To be therapeutically effective, a carrier must meet the following basic requirements:

i. It must not only show specificity for the target site but it must also reach that site in vivo.

ii. It should possess appropriate biochemical properties so that cytotoxic agents can be bound to it without detrimentally affecting either its specificity or the reactivity of the

agent.

iii. The carrier should be nontoxic, although selective preferential toxicity for the target site might be beneficial.

iv. Carrier-cytotoxic agent complexes should not home in appreciable amounts to nontarget areas, and the carrier must not be inactivated during transit from the point of administration to the target site.

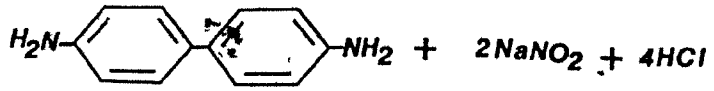
v. The transported toxic agents should reach the target area in active form.

By virtue of their unique specificity and high affinity for antigen, antibodies are particularly attractive as selective carriers of toxic agents. Indeed, Ehrlich in the early 1900's, was fascinated by the possibility of using diphtheria toxin bound to antitumor antibodies as a "magic bullet" against malignant diseases. The classic investigation by Landsteiner (1946) on methods of linking haptens to macromolecules led to the binding of various cytotoxic agents to antibodies. Agents that have been bound covalently to antibodies with some success in inhibiting tumor growth include MTX (Kulkarni, et al., 1981; Mathe et al., 1958), diphtheria toxin (Moolten et al., 1975), chlorambucil (Tai et al., 1976), trenimon (Froese et al., 1976), daunomycin and adriamycin (Hurwitz et al., 1975), ricin (Moolten et al., 1976).

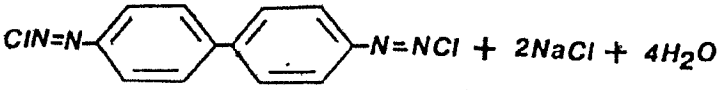
F. METHODS OF COUPLING MTX TO ANTIBODIES

(i) DIAZOTIZATION

The coupling of MTX to antibody molecules was usually achieved

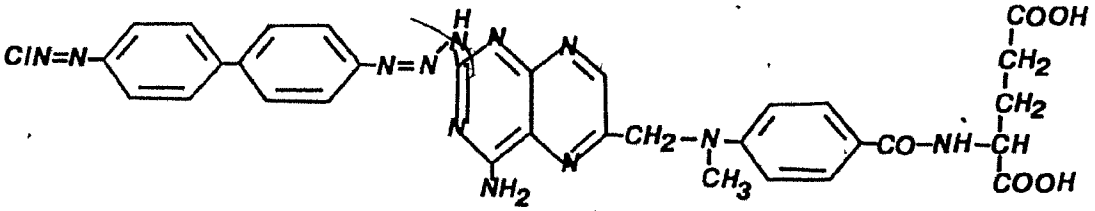
1 

Benzidine



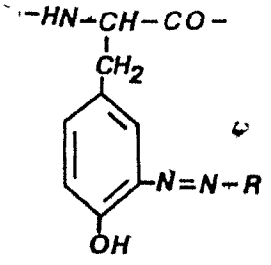
Tetrazo-benzidine-chloride

2 Tetrazo-benzidine-chloride + MTX



Tetrazobenzidine-MTX complex $\left(R-N=N-Cl\right)$

3 Tetrazobenzidine-MTX complex $\left(R-N=N-Cl\right)$ + Tyrosine residue of IgG



IgG-Tetrazobenzidine-MTX conjugate

through the following three steps (DeCarvalho et al., 1964):

i. diazotization of benzidine

ii. coupling of methotrexate to diazotized benzidine

iii. coupling of the tetrazobenzidine - MTX complex formed in step (ii) to the IgG molecule.

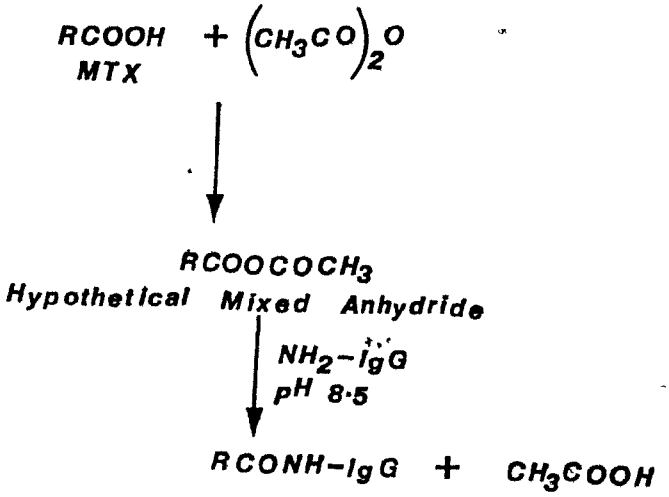
The inherent advantages of using diazonium salts for linkage of agents to immunoglobulins include the ability to carry out the reaction in an aqueous medium at pH values between 5 and 8, the fact that no additional charge is introduced in the product, and the rapidity and completeness of the reaction at low temperature. Furthermore, the bonds formed by diazonium salts are easily cleaved (Avrameas et al., 1978; Cohen, 1974). Thus, the diazo linkage might be preferred if dissociation of the drug at target sites is desired. However, it has been found that it is difficult to control the side reactions associated with the use of diazonium compounds leading to extensive precipitation in the reaction mixture (DeCarvalho et al., 1964; Robinson et al., 1973). Also, diazonium salts react preferentially with amino acids like tyrosine or histidine, that is, residues that may be present in substantial numbers at or near the antigen binding site (Avrameas et al., 1978). This could lead to considerable loss of antibody activity. Furthermore, since the 2,4 diamino pyrimidine configuration of MTX is required for maximum cytotoxic effect, a diazo coupling reaction involving one or both of these amino groups would be expected to negate the cytotoxic

properties of MTX.

These disadvantages notwithstanding, using the diazo reaction, Mathe et al., (1958) bound MTX to a hamster anti-mouse L1210 globulin and reported prolonged survival of tumor-inoculated mice given injections of the conjugate. Injections of comparable amounts of antitumor globulin, MTX, or MTX bound to normal hamster globulin all failed to inhibit tumor growth.

(ii) MIXED ANHYDRIDE FORMATION

An amide bond between MTX and IgG should also be achievable by a mixed anhydride method. Burstein and Knapp (1977) heated MTX with acetic anhydride to form an intermediate which was then reacted with IgG at pH 8.5 and at room temperature.

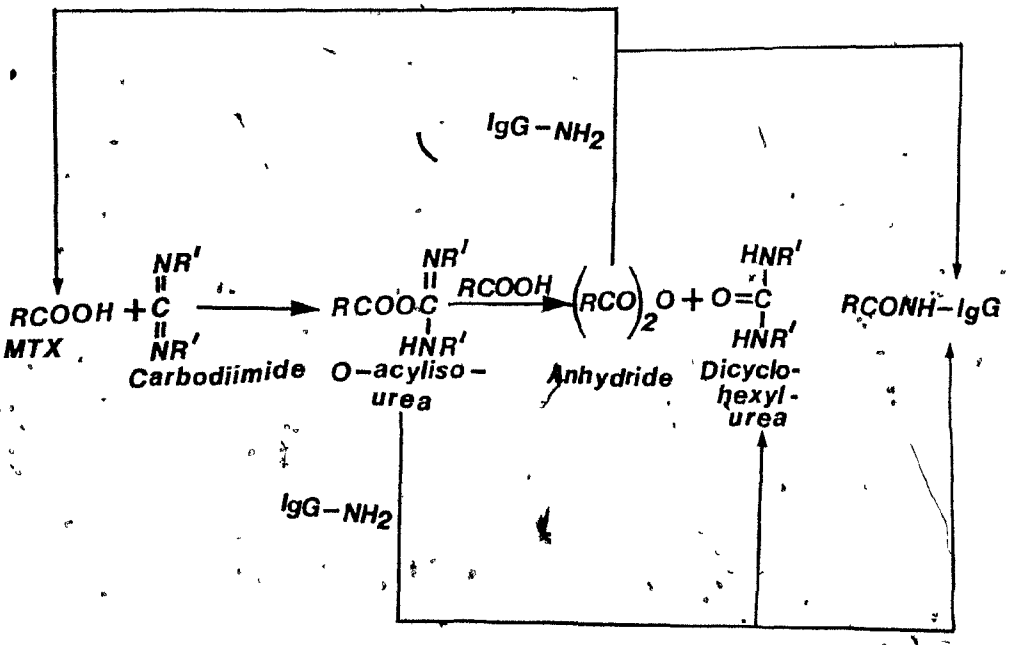


They reported production of active conjugates of MTX and an anti-mouse ovarian carcinoma antibody with a molar incorporation of 15 to 120 mole of MTX per mole of IgG depending on the reaction conditions, and also with retention of antibody activity. This conjugate was reported to prolong survival in tumor-bearing mice compared to those treated with free drug, antibody alone, a mixture of both or MTX coupled to nonspecific IgG. However, these results have not been reproduced in other laboratories. Latif et al., (1980) reported loss of antibody activity when IgG was reacted with the product obtained by treating MTX with acetic anhydride. Kulkarni et al., (1981) retained antibody activity but obtained only a small amount of MTX bound to IgG (2-3 mole of MTX per mole of IgG). Moreover, neither the MTX-IgG conjugate nor the product resulting from the reaction of MTX with acetic anhydride inhibited DHFR in vitro even after incubation of the product at pH 8 for up to 48 hr to allow hydrolysis.

(iii) CARBODIIMIDES

Carbodiimides first introduced for peptide synthesis by Sheehan and Hess (1955) and for oligonucleotide synthesis by Khorana (1953) have now been used to link MTX to antibodies, the most widely used being dicyclohexyl carbodiimide and the water soluble 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (ECDI). The conditions of the reaction are very simple. IgG, MTX in excess and the reagent are simply stirred together in an aqueous solution at room temperature or 4°C. The carbodiimide activates the carboxylic acid group of MTX

by formation of an O-acylisourea which then reacts with the amino group of the antibody molecule by nucleophilic acyl substitution. Alternatively, the O-acylisourea could react with a second carboxylic acid group of MTX to form a symmetrical anhydride which in turn could react with an amino group in the antibody molecule to form a stable peptide bond.

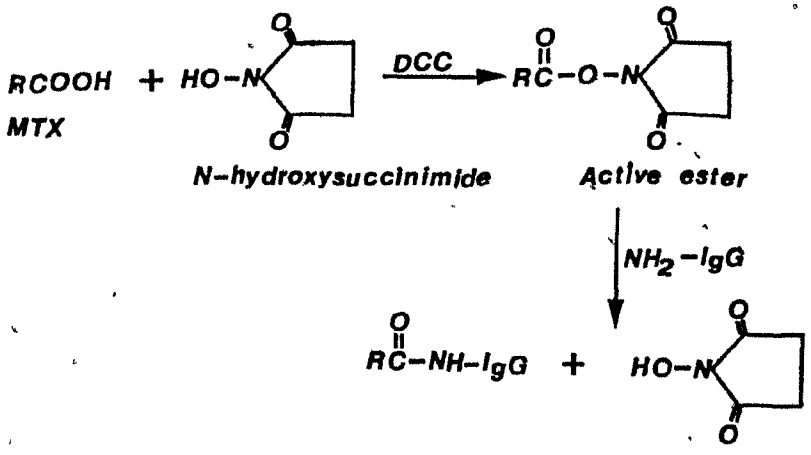


Using the carbodiimide method, Robinson et al., (1973) linked MTX to anti L1210 cell antibodies and then treated mice inoculated intraperitoneally with L1210 cells with the conjugates; they survived for more than 75 days, whereas mice inoculated with MTX and antibody unlinked survived only about 27 days. This method has certain disadvantages. A major disadvantage of this method is the presence of both carboxyl and amino groups in the antibody molecule as well as in

the MTX molecule which can result in extensive polymerization. The reactive O-acylisourea can undergo extensive rearrangement to form the nonreactive and sparingly soluble N-acylisourea which results in the reduction of the yield.

(iv) THE ACTIVE ESTER METHOD

A major disadvantage of the carbodiimide method is the rearrangement of the O-acylisourea to form the non-reactive N-acylisourea. This can be reduced dramatically by performing the coupling in the presence of a nucleophile which will react very rapidly with the O-acylisourea to give an acylating agent which is still reactive enough for aminolysis but which is more discriminating and does not lead to rearrangement or other side reactions. The first reagent to be used in this way and introduced for peptide synthesis in 1966 was N-hydroxysuccinimide (Wunsch and Drees, 1966; Weygand et al.,



1966). The active ester of MTX is normally prepared using equimolar amounts of MTX, N-hydroxysuccinimide and dicyclohexyl carbodiimide in dimethyl formamide. The active ester formed is stable at 4°C for several weeks under nitrogen. Kulkarni et al., (1981) have demonstrated that this active intermediate method is more efficient in coupling MTX to antibodies than other methods that are currently being used. They showed that conjugates prepared by this method retained up to 70% antibody activity at drug incorporation levels of 12 mole of drug per mole of IgG. Conjugates of MTX-anti EL4 IgG inhibited DHFR and tumor cells in vitro as well as prolonged survival of EL4 lymphoma bearing mice compared to drug alone, drug plus antibody unlinked and drug coupled to normal IgG (Kulkarni et al., 1981).

G. MEMBRANE TRANSPORT OF PROTEINS AND ANTIBODIES

(1) ENDOCYTOSIS

Endocytosis is a widespread cellular function that regulates the uptake of exogenous molecules from the cell's environment via plasma-membrane-derived vesicles and vacuoles. Endocytic activity has always been divided into two categories-phagocytosis, or "eating", and pinocytosis, or "drinking". Most investigators use the term phagocytosis to describe the uptake of large particulates, i.e. those visible by light microscopy and possibly some viruses. Uptake occurs by close apposition of a segment of plasma membrane to the particle's surface, excluding most, if not all, of the surrounding fluid. The term pinocytosis is used to describe the vesicular uptake of everything else, ranging from small particles (lipoproteins, ferritin,

colloids, immune complexes) to soluble macromolecules (enzymes, hormones, antibodies, yolk proteins, toxins), to fluid and low molecular weight solutes. The terms fluid and adsorptive endocytosis (or pinocytosis), initially used by several workers are useful in describing and analyzing the uptake of many biologically important materials, such as those cited above. These terms point to the fact that substances can enter the cell in the fluid content of an endocytic vesicle (fluid-phase pinocytosis) and/or be bound to the inner aspect of the vesicle membrane (adsorptive pinocytosis). In both types, the rate of uptake is determined by the size of the vesicle and the rate of its formation. In fluid-phase endocytosis, uptake is directly related to the concentration of solute in the extracellular fluid whereas in adsorptive endocytosis, uptake in addition depends on the number, affinity and function of the cell surface binding sites. Adsorptive uptake is both a selective and concentrating device whereby cells can interiorize large amounts of a specific solute without ingesting a correspondingly large volume of solution.

The cytoplasmic surface of many endocytic vacuoles is smooth, but electron micrographs show that most cell types contain an additional population of vesicles in which regularly spaced, short bristles protrude into the cytoplasm. Coated vesicles were first noted by Roth and Porter (1964) and by Droller and Roth (1966) in oocytes, and by Fawcett (1964) in reticulocytes. Biochemically, the coat consists primarily of clathrin (Pearse, 1976), a 180-K dalton protein which has

been extensively purified and reassembled into cages in vitro (Keen et al., 1979; Woodward and Roth, 1979; Ungewickell and Branton, 1981; Crowther, and Pearse, 1981; Kirschhansen and Harrison, 1981; Merisko et al., 1982). Other coat-associated proteins have been identified, the "light chains" (with molecular weights of 30,000 and 32,000 daltons) as well as an additional polypeptide of 100,000 daltons (Ungewickell and Branton, 1981). The light chains are believed to attach in vitro to the arms of clathrin trimers (known as triskelions) (Ungewickell and Branton, 1981; Crowther and Pearse 1981; Kirschhansen and Harrison, 1981), while the 100-K dalton protein may be important in mediating clathrin attachment to membranes (Unanue et al., 1981). Because clathrin can form spherical or hemispherical cages in vitro, its function may be to drive the endocytosis or budding of the segment of plasma membrane on which clathrin assembles (Silverstein et al., 1977; Pearse, 1983; Steinman et al., 1983; Pastan and Willingham, 1981; Brown et al., 1983; de Duve, 1983). Pearse and Bretscher (1981) have postulated that clathrin acts as a molecular sieve that selects specific proteins to be interiorized.

Steinman et al., (1974) have shown that the rate of solute uptake by pinocytosis in mouse fibroblasts has a Q_{10} of 2.7, an activation energy of 17.6 kcal per mole, and is directly proportional to the incubation temperature from 2° to 38°C whereas particles bind to the surfaces of phagocytic cells at 4°C but are not ingested unless the temperature of the incubation medium exceeds some critical threshold (18-21°C). Thus, there appears to be a critical thermal transition

for cells below which phagocytosis of large particles cannot occur. Binding of ligands to cells can stimulate pinocytic activity. Edelson and Cohn (1974) found that Con A increased the uptake of fluid-phase markers threefold in mouse macrophages and that mannose and glucose blocked the lectin's effect. They identified the Con A on the cell's surface and on the inner aspect of the endocytic vacuole membrane. The fact the Con A is bivalent or possibly multivalent in its interaction with cell-surface saccharides seems to be significant because succinylated Con A which behaves as a univalent ligand in several mouse cells, does not stimulate pinocytosis even though it is interiorized in an adsorptive fashion.

B lymphocytes rapidly pinocytose antiIgG molecules bound to their cell-surface immunoglobulins (Taylor et al., 1971; Unanue et al., 1972; Gonatas et al., 1976); again, the bivalent nature of the anti immunoglobulin is important since univalent anti immunoglobulin fragments are not rapidly interiorized (Taylor et al., 1971; Unanue et al., 1972). In contrast to the effects of the endocytosis of indigestible materials on enzyme secretion, the endocytosis of digestible materials appears to regulate the levels of intracellular lysosomal enzymes. Phagocytosis of red blood cells or pinocytosis of serum proteins induces the synthesis of lysosomal enzymes in mouse macrophages and leads to a tenfold increase in their intracellular content (Axline and Cohn, 1970). The uptake of latex or sucrose has no corresponding stimulating effect, which suggests that the products of intracellular digestion are responsible for this increase in enzyme

synthesis (Axline and Cohn, 1979).

(ii) RECYCLING OF PLASMA MEMBRANE DURING ENDOCYTOSIS

The idea of recycling of plasma membrane was initially proposed by Palade (1956) following his initial electron microscopic observations of endoplasmic reticulum. Until recently, it has been difficult to obtain evidence supporting this concept. The findings of Steinman et al., (1972) provide experimental support for the membrane-recycling hypothesis. Steinman et al., showed that the total surface area of plasma membrane, pinocytic vesicle membrane, and secondary lysosomal membrane of mouse fibroblasts each remains constant despite extensive interiorization of the cell's surface membrane during pinocytosis. These results led Steinman et al., (1972) to propose that interiorized membrane is recycled back to the cell surface intact. Extensive degradation and resynthesis, at least for most membrane components were considered unlikely in view of the rapidity of the process, the considerable metabolic load this would impose and the failure to detect such rapid degradation in most studies of plasma membrane turnover.

It has been shown that a number of ligands of great physiological interest, such as low-density lipoproteins (LDL) Goldstein et al., 1979; Goldstein et al., 1982), peptide hormones (King and Cuatrecasas, 1981) and transport proteins, e.g., transferrin (Regoeczi et al., 1982) enter the cell by receptor-mediated endocytosis (Goldstein et al., 1979), by binding to specific receptors on the cell membrane and are then internalized by concentration in clathrin-coated pits and

vesicles. In the majority of cases so far studied, the internalized ligand has been shown to be delivered to lysosomes, but delivery to other destinations e.g. the Golgi complex (Posner et al., 1981) or GERL (Golgi apparatus-endoplasmic reticulum-lysosomes) (Willingham and Pastan, 1982) has also been documented. According to the classical lysosome concept (de Duve and Wattiaux, 1966) the endosome, i.e., the pinosome or phagosome, fuses directly with a primary or secondary lysosome. Evidence has been obtained on several systems [fibroblasts in culture (Pastan and Willingham, 1981) and hepatocytes (Wall et al., 1980)] indicating that the incoming vesicles fuse with an intermediate or prelysosomal compartment prior to delivery of the internalized ligand (α_2-macroglobulin and asialoglycoproteins, respectively) to lysosomes. There is now both morphological (Pastan and Willingham, 1981; Wall et al., 1980; Courtoy et al., 1982) and biochemical (cell fractionation) (Courtoy et al., 1982) evidence for the existence of a distinctive intermediate endosome or prelysosomal compartment, which has been called a "receptosome" (Pastan and Willingham, 1981) or a "sorting vesicle" (Goldstein et al., 1982).

The existence of such a prelysosomal or sorting compartment provides a means by which surface membrane constituents such as LDL (Goldstein et al., 1982) and asialoglycoprotein (Schwartz et al., 1982) receptors can recycle directly back to the cell surface and escape lysosomal digestion after which the ligand can be delivered to the lysosome and the receptor can return to the cell surface. This, however, requires that the ligand dissociate from its receptor in

transit through the receptosome or sorting vesicle. One mechanism to explain how this takes place has been provided by the finding (Tycko and Maxfield, 1982; Maxfield, 1982) that this intermediate compartment (which lacks lysosomal enzymes) has a low pH (approx. 5.0). The low pH would facilitate dissociation of many ligands from their receptor because a number of ligands [e.g. LDL (Goldstein et al., 1982; Anderson et al., 1982), asialoglycoproteins (Wall et al., 1980; Schwartz et al., 1982), epidermal growth factor (EGF) (Haigler et al., 1980) and insulin (Posner et al., 1981)] undergo rapid dissociation from their receptors when the pH drops below 5.5. Thus, the intermediate endosome compartment may serve as an "acid wash" (Palade, 1982) which promotes ligand-receptor dissociation and at the same time cleanses internalized plasma membrane of extraneous molecules taken up from the extracellular environment nonspecifically (e.g. by electrostatic interaction) before it is recycled back to the cell surface. It has recently been shown (Galloway et al., 1983) that the membrane of endosomes, like that of lysosomes (Ohkuma et al., 1982; Schneider, 1981), contains an ATP-driven proton pump believed to be responsible for maintaining the low pH.

H. MECHANISMS OF PROTEIN DEGRADATION IN CULTURED CELLS

At least two separate mechanisms have been implicated in protein turnover occurring in cultures of mammalian cells. For convenience Knowles and Ballard have called these an A mechanism, characterized by marked variation in activity, and a B mechanism, which maintains a relatively constant rate. These two mechanisms can be distinguished

by their different sensitivities both to alterations in the cellular environment and to inhibitors of proteolysis. For example, the A mechanism is immediately stimulated when cultured cells are placed in a serum-deficient medium (Knowles and Ballard, 1976; Hershko and Tomkins 1971; Poole and Wibo, 1973; Amenta et al., 1977a; Hershko and Ciechanover, 1982; Amenta and Brocher, 1981) and in turn, this induced cellular proteolysis can be rapidly inhibited by cycloheximide, (Hershko and Tomkins, 1971; Amenta et al., 1977a; Auricchio et al., 1969; Amenta et al., 1977b), insulin (Knowles and Ballard, 1976; Amenta et al., 1977a; Auteri et al., 1983) and microtubular poisons (Amenta et al., 1977b; Amenta et al., 1976; Seglen, 1983). In contrast, these agents in short-term experiments have little or no effect on the B mechanism. Studies on isolated liver cells (Seglen, 1975; Seglen, 1976; Seglen, 1977; Seglen and Reith, 1976) have demonstrated that NH_4Cl and the salts of organic bases are potent inhibitors of proteolysis in the A system. A similar inhibition has been observed in cultured fibroblasts (Amenta et al., 1977a; Wibo and Poole, 1974; Glimelius et al., 1977; Amenta et al., 1978). Detailed studies by Ohkuma and Poole (1978) indicate that these latter inhibitors are concentrated in the acidic lysosome and effect an increase in the pH sufficient to inhibit the action of the acid proteases within the vacuole, suggesting that a common mechanism of action is involved for this group of agents. These studies suggest that the variable A pathway represents protein degradation occurring in the lysosomal system.

While it is reasonably clear that the autophagic mechanism can, under appropriate conditions, be activated in both in vitro and in vivo systems and contribute significantly to the degradation of proteins, the role of the lysosomal system in basal proteolysis (B mechanism) remains controversial. In order to analyze data on this subject it is necessary to ascertain that the experiments were conducted under conditions in which the autophagic mechanism was not in an activated state. The cells being studied should be maintained in fresh growth medium containing an adequate amount of serum to suppress proteolysis and also, agents such as insulin or vinblastine, that are known to inhibit induced autophagic proteolysis, must be seen not to affect the rate of cell protein degradation. While this would be most difficult to achieve in organ perfusion experiments as well as experiments on direct isolates of cells, where the initial preparatory steps always activate lysosomal proteolysis, carefully controlled experiments with monolayer cultures offer a reasonable possibility of attaining a controlled level of basal proteolysis in these cells. Studies on rat fibroblasts carefully maintained under basal conditions showed no suppression of proteolysis with either insulin or vinblastine, yet a 22% inhibition when chloroquine was added to the culture (Amenta et al., 1977a). NH_4Cl also produced 10-30% inhibition of basal proteolysis in this system (Amenta et al., 1978a; Amenta and Brocher, 1980a; Amenta and Brocher, 1980b). This small but significant inhibition of basal proteolysis affected by these salts of weak bases suggested that these agents not only completely block any

induced proteolysis (autophagic-mechanism), but also concurrently suppress a small component of basal proteolysis. Poole and co-workers (1978b), observing a similar differential inhibition of proteolysis in macrophages and rat fibroblasts treated with chloroquine, have also concluded that two differential lysosomal pools are involved, one in the basal degradation of cellular proteins, and a second involving degradation of proteins entering the lysosomal system via autophagy.

That a lysosomal mechanism complements basal protein degradation can also be demonstrated by inhibiting protein degradation in cell cultures with cycloheximide. When exposed to this agent alone, fibroblasts in growth medium show a 15-20% decrease in the basal degradation rate of cell protein (Amenta et al., 1977b; Amenta et al., 1978). When the cells were exposed concurrently to both cycloheximide and NH_4Cl, the NH_4Cl contributed very little to the inhibition already caused by cycloheximide, suggesting that both agents were affecting the complementary lysosomal mechanism. It has been suggested that the inhibitory effect of cycloheximide on proteolysis is not related to its inhibition of protein synthesis (Rez et al., 1976; Gunn, 1978). Thus, it appears that basal proteolysis involves at least two mechanisms, one involving acid proteases in lysosomes, and another involving neutral proteases probably located in the cytoplasm. Neff et al., (1979) have proposed that slow-turnover proteins are degraded by the lysosomal acid proteases and the fast turn-over proteins are degraded by neutral proteases.

I. DEGRADATION OF IgG BY CELLS AND CELL EXTRACTS

In view of the reports that tumor cell extracts have extensive proteolytic activity (Maver et al., 1945, 1948; Purr, 1934; Shamberger et al., 1971; Schersten et al., 1971) and the findings of Sylven et al., (1965, 1968) that lysosomal enzymes were detected in the extracellular fluid of various types of tumors, attempts have been made to determine if tumor cells are capable of degrading the IgG molecule. Engers and Unanue (1973) complexed [^{125}I] rabbit IgG antimouse immunoglobulin to the surface-bound immunoglobulin of B lymphocytes and investigated its fate in culture. They observed that early during culture a small amount of antiimmunoglobulin with a sedimentation constant greater than IgG was released into the culture supernatants. Most of the remaining immunoglobulin was catabolized. Examinations of culture medium and cell supernatants identified the ^{125}I bound to amino acids or to small protein fragments. This metabolism of the rabbit IgG against mouse immunoglobulins was temperature-dependent and also specific for that fraction of the rabbit IgG capable of interacting with the surface immunoglobulin present on mouse B lymphocytes. They therefore concluded that B lymphocyte sheds a small percentage of the immune complex and internalizes and effectively degrades the remainder. A similar conclusion was reached by Fish et al., (1974) and Keisari and Witz (1978, 1979) after observing that TA3 and EL4 tumor cells, respectively, could degrade their surface membrane bound antibodies to dialyzable products which could not be precipitated with ammonium sulfate. In an earlier report, Keisari and Witz (1973) noted that

lysosomal extracts derived from various mouse tumors degraded mouse immunoglobulins upon in vitro incubation at pH 3.6 or at pH 3.8. IgG_1 was more sensitive than IgG_2 to the proteolytic activity of this lysosomal extract. This agrees with the results obtained by others showing differences in susceptibility of various immunoglobulin subclasses to proteolytic digestion (Virella and Parkhouse, 1971; Gergely et al., 1972). Along these lines, Motas and Ghetie (1969), Ghetie and Motas (1971) as well as Lo Spalluto et al., (1971) have found that lysosomal fractions rich in cathepsin activity partially degrade IgG, yielding Fab' and Fc fragments. While the Fc fragment was easily degraded to smaller fragments, the Fab' moiety was resistant to degradation. The results of Sobczak and de Vaux Cyr (1971) which showed that they were able to elute Fab' and Fc fragments, in addition to intact IgG_2 molecules from in vivo grown SV40 induced hamster tumors imply that immunoglobulin degrading cathepsins of tumor origin function in vivo.

In a more detailed study, Schneider et al., (1981) incubated cultured rat fibroblasts with IgG doubly labeled by ^3H-acetylation and conjugation with fluorescein (FAC-IgG). The fibroblasts took up the FAC-IgG continuously for at least 72 hr. They returned the major part of the internalized IgG back to the medium in the form of breakdown products of very low MW. Gel filtration and immunological analysis of cells and medium at various times indicated that essentially all the FAC-IgG molecules taken up underwent digestion of their Fc moiety but that the Fab moieties of only about 75% of the molecules was

degraded. The rest remained stored intracellularly in the form of $F(ab')_2$-type fragments that slowly dissociated into Fab'-type fragments. When FAC-IgG was incubated in vitro in the presence of a hepatic lysosomal extract, complete digestion of the Fc part likewise occurred, but the Fab' part of most if not all the molecules proved resistant to breakdown, and remained as Fab'-type fragments. Cell fractionation experiments demonstrated that the compartment in which FAC-IgG and its digestion residues was stored: (a) showed a density distribution pattern in a sucrose gradient identical to that of the lysosomal marker N-acetyl-β-glucosaminidase and clearly dissociated from that of the Golgi marker galactosyltransferase, and (b) accompanied the lysosomal marker in its density shift induced by exposure of the cells to chloroquine. Thus, they concluded that storage and processing of FAC IgG by rat fibroblasts occur in a single, digestively active compartment of lysosomal nature, and that resistance to digestion of certain Fab'-type fragments accounted largely for the inability of the lysosomal enzymes to completely digest the FAC-IgG taken up.

The mechanism and site of IgG degradation in vivo, is not well understood, but after injecting [^{125}I]IgG_{2a} i.v into rats, Fukumoto and Brandon (1981) showed a close relationship between the binding of IgG_{2a} to the liver, spleen and lymph nodes and its catabolism. Intermediate products and metabolites of IgG_{2a} were also found intracellularly in the spleen and lymph nodes.

J. OBJECTIVES

Robinson et al., (1973) had shown that MTX linked to anti L1210 cell IgG by the carbodiimide method prolonged the survival of mice inoculated intraperitoneally with L1210 cells more than those given unlinked antibody and MTX. Chu and Whiteley (1977), however, demonstrated that MTX linked to bovine serum albumin (BSA) was as effective as free MTX in prolonging the survival of mice inoculated intraperitoneally with L1210 cells. Kulkarni et al., (1981) have compared the ability of free MTX, MTX linked to normal rabbit IgG (NRG), AELG alone, AELG mixed with free MTX and AELG linked to MTX by the active ester intermediate method, in prolonging the survival of mice inoculated intraperitoneally with EL4 cells. They found that even though MTX linked to NRG prolonged the survival of mice more than free drug, MTX-AELG was much more effective in prolonging the survival of mice. This finding by Kulkarni et al., clearly established the superiority of MTX-antibody conjugate in inhibiting tumor growth in vivo; however, its mechanism of action has not been established.

The objective of this thesis is to determine the factors that contribute to the superior effectiveness of the specific antibody-MTX conjugate in inhibiting tumor growth in vivo. For example, the superior therapeutic effectiveness could be due to (a) greater delivery of the MTX-antibody conjugate to the tumor cell and/or (b) longer plasma half life of the conjugate, thereby acting as a depot from which more drug is continuously delivered to the tumor. Using the EL4 lymphoma and Human melanoma M21 cells, these have been investigated by comparing the in vitro and in vivo uptake and level of

accumulation of (a) free MTX, (b) MTX-NRG conjugates (c) MTX-antitumor IgG conjugates. The rate of efflux of free or conjugated MTX from the cells in an environment depleted of drug as well as the ability of the cells to release free MTX or active MTX-containing fragments that can inhibit DHFR have also been examined.

MATERIALS AND METHODS

A. MATERIALS

Freund's complete adjuvant was obtained from Difco Laboratories, Detroit, MI, USA. DEAE-cellulose DE52 and DE32 were purchased from Whatman, England. Sephadex G200 was the product of Pharmacia Fine Chemicals, Uppsala, Sweden. Fluorescein-conjugated antibody against mouse or rabbit 7S globulin was obtained from Hyland Laboratories, Los Angeles CA, USA. Unlabeled MTX, Sodium dodecyl sulfate, Chloroquine, Leupeptin, Bovine Liver DHFR, NADPH, Dihydrofolic acid, Antipain, N α-p-tosyl-L-lysine chloromethyl ketone and Pepstatin were purchased from Sigma Chemical Co., St. Louis, MO., USA. [^{3}H]MTX, Aquasol-2 and Oxifluor-H_2O were the products of New England Nuclear, Boston, MA, USA. Dimethylformamide and N-hydroxysuccinimide were obtained from Aldrich Chemical Co., Milwaukee, WI, USA. Dicyclohexylcarbodiimide was purchased from Eastman Organic Chemicals, Rochester, NY, USA. Biogel P100, High purity acrylamide, Bis-acrylamide, Ammonium persulfate and Bovine plasma albumin were the products of Bio-Rad Laboratories, Richmond, CA, USA. RPMI 1640 and Fetal calf serum were obtained from Flow Laboratories, McLean, VA, USA. The Coulter counter Model "Z"$_f$ was purchased from Coulter electronics, Inc., Hialeah, FL, USA and the Oxymat Model IN 4101 was the product of Intertechnique, Paris, France. Cellulose coated thin layer chromatography glass plates were obtained from Brinkman Instruments Inc., Westbury, NY, USA.

All other chemicals and solvents used were reagent grade or the

highest available quality.

B. MICE, TUMORS AND METHODS OF TRANSPLANTATION

(i) EL4 LYMPHOMA

The murine EL4 lymphoma was obtained from the Chester Beatty Research Institute, London, U.K. and maintained by serial intraperitoneal passage in syngeneic female C57BL/6J mice purchased from the Jackson Laboratory, Bar Harbor, ME, USA. The mice were housed in groups of 5 in plastic cages in an air-conditioned room maintained between 20 and 25°C. The diet consisted of Purina Laboratory Chow (Ralston Purina of Canada LTD, Woodstock, Ont.) and tap water, ad lib.

(ii) HUMAN MELANOMA M21 CELL LINE

The human melanoma M21 cell line was obtained from the Scrips Research Institute, La Jolla, CA, USA and maintained by serial subcutaneous transplantation in the flank of nude mice of BALB/c background (Harlan Sprague-Dawley Inc., Madison, WI, USA). A few passages in vivo stabilized the line in this nude mouse host so that now transplanting 2 x 10^6 M21 cells into 8-week-old mice always leads to the formation of a palpable tumor between the 7th and 10th day followed by steady progressive growth which does not vary significantly from mouse to mouse. The nude mice were kept in isolation cages in a room under positive pressure with filtered air and given ad lib sterile water and autoclaved Purina Laboratory Chow.

The M21 cell line is also maintained in vitro, by weekly passage of 10^6 cells into tissue-culture flasks containing RPMI 1640

supplemented with 10% fetal calf serum.

C. HARVESTING OF TUMORS

(i) EL4 LYMPHOMA

Harvesting of EL4 lymphoma from passage mice was performed as follows:

(a) The abdominal skin of the ascites tumor-bearing mouse was cleaned with 70% ethyl alcohol, and a sterile 18-gauge needle was inserted into the peritoneal cavity.

(b) Ascites fluid dripping from the free end of the needle was collected in sterile centrifuge tubes containing about 20 ml of sterile PBS.

(c) The total volume of the cell suspension was measured and a total cell count was performed.

(d) The cell suspension was centrifuged at 300g for 15 to 20 minutes.

(e) Tumor cells were resuspended in sterile PBS or RPMI 1640 at desired concentrations.

(ii) HUMAN MELANOMA M21 CELL LINE

Harvesting of M21 cells from passage nude mice was performed by removing the tumor under sterile conditions, then dissociating it mechanically by passage through a wire mesh (400 holes/cm^2). Nonviable cells were removed by differential flotation on Ficoll-Hypaque and after washing, resuspended in PBS or RPMI 1640 at the desired concentration.

Tumor cells in tissue culture were harvested after incubation

with a calcium and magnesium free EDTA solution, washed twice, and resuspended in PBS or RPMI 1640.

D. CELL COUNTS AND VIABILITY TESTS

Tumor cell counts were performed with a Spencer haemocytometer or with a Coulter Counter.

Viability of the cells was determined by the exclusion of 1% (w/v) trypan blue in distilled water diluted 10 times with 0.9% (w/v) saline.

E. PRODUCTION OF RABBIT ANTI EL4 LYMPHOMA ANTIBODY (AELG)

Six rabbits at a time were each injected i.m. in both flanks and both shoulders with a total of 4×10^8 freshly obtained EL4 cells, which had been washed 3 times with PBS at 4°C ($\geq$98% trypan blue impermeable). For injection, the cells were suspended in 4 ml of PBS and mixed with an equal volume of Freund's complete adjuvant. The injections were repeated after 1 week. Beginning 1 week after this second set of injections, the rabbits were injected i.m. twice a week with 10^8 cells/rabbit with no adjuvant. Three days after the tenth such injection, 5 ml of blood were obtained from the ear vein of each rabbit (test bleeding). The resulting test sera were separately absorbed with homogenates of normal mouse tissues and assayed thereafter by immunofluorescence for the presence of specific anti EL4 antibody, as described below. Rabbits showing titers of 1:64 or greater were exsanguinated. The pooled sera were inactivated at 56°C for 30 min and then successively absorbed with washed homogenates of liver, lungs, kidneys and spleen from normal adult C57BL/6J mice.

Absorption with each homogenate (homogenate: serum ratio, 1:3 by volume) was carried out with gentle shaking for 4 hr at 37°C followed by another 8 hr period at 4°C. Sera were separated from homogenates by centrifugation (20,000 x g) for 2 hr at 4°C. Absorptions were repeated (usually twice with liver, lung and kidney and 3 to 4 times with spleen homogenate) until the serum reacted on immunofluorescence assay with EL4 cells only and not with either cryostat sections of normal C57BL/6J mouse tissues or suspensions or smears of C57BL/6J lymphoid cells derived from lymph nodes, spleen and thymus; B16 melanoma cells; 2 lines of AKR/J lymphoma cells; and Ehrlich ascites tumor cells maintained in BALB/CJ mice.

F. PRODUCTION OF RABBIT ANTIMELANOMA M21 ANTIBODIES (AHMG)

For production of rabbit antimelanoma M21 antisera, washed, trypan blue impermeable M21 melanoma cells were mixed with 2 ml of Freund's complete adjuvant and injected i.m. into adult New Zealand white rabbits (4×10^8 cells/rabbit). Starting one week later, 11 injections were given without adjuvant over a period of 4 weeks. Three days after the final injection, animals were test-bled and after serial absorptions as described below, the resulting sera were tested by immunofluorescence for specific melanoma activity. Animals whose sera showed a titer of 1:64 or greater were bled to obtain a maximum quantity of immune sera. The serum was then heat inactivated and repeatedly absorbed with group AB, RH^+ red cells, and homogenates of normal human liver, lung, kidney and spleen. The absorptions were repeated until the sera would react only with the immunizing melanoma

cells and not with normal human skin fibroblasts, human peripheral blood lymphocytes or other normal adult human tissues. The specificity and reactivity of the antihuman melanoma sera were determined according to the methods outlined by Ghose et al., (1975). In some experiments, as indicated, the rabbit anti M21 sera were used after absorptions with only AB, RH^+ red cells. The objective was to provide additional antigenic sites for binding of conjugates.

G. MONOCLONAL ANTIBODY 225.28S TO A HIGH MOLECULAR WEIGHT MELANOMA ASSOCIATED ANTIGEN (MAB).

This monoclonal antibody was kindly supplied by Dr. Soldano Ferrone (Dept. of Microbiology and Immunology, New York Medical College, Valhalla, NY 10595). Its preparation and the serological and immunochemical characterization of its specificity have been described (Imai et al., 1981; Wilson et al., 1981). The MAA to which the MAB is directed against consists of a 280 K dalton glycoprotein noncovalently associated with other glycoproteins ranging from 300 K to 700 K daltons. It is expressed by the vast majority of malignant melanomas, nevi and a small proportion of squamous and basal cell carcinomas. It has not been detected in normal tissues and its expression is not associated with synthesis of melanin. Though this MAA is expressed in primary, metastic or recurrent melanomas, there is a quantitative variation even in a given tumor cell population. Monoclonal antibody 225.28S was purified from ascites fluid by sequential ion exchange chromatography and gel filtration.

H. FRACTIONATION OF SERA

All sera and MAB preparations were brought to 33% saturation by adding saturated aqueous ammonium sulfate. The precipitate of MAB 225.28S, AELG, $AHMG_R$, $AHMG_{R+T}$ or of NRG was dissolved in PBS and the ammonium sulfate was removed by repeated dialysis against PBS.

I. MEMBRANE IMMUNOFLUORESCENCE ASSAY

Membrane immunofluorescence assay of tumor cells and other control preparations were performed by the sandwich method as described by Ghose et al., (1977), using fluoresceinated goat antibody against mouse or rabbit 7S globulin. The end point of the titre is taken as visible staining of 50% of the cells exposed to the antibody or its conjugate.

J. PREPARATION OF MTX-IMMUNOGLOBULIN CONJUGATES

MTX was conjugated to IgG by an active ester intermediate method described by Kulkarni et al., (1981). 0.1 mmol of unlabeled MTX was mixed with [^{3}H]MTX to give a specific activity of 5 mCi/mmol and dissolved in 1.0 ml of dimethylformamide. 0.1 mmol of N-hydroxysuccinimide in 0.5 ml of DMF was added with stirring, followed by 0.1 mmol of dicyclohexylcarbodiimide in 0.5 ml of DMF. The reaction mixture was stirred for 1 hr at room temperature and then for 18 hr at 4°C in the dark and under anhydrous conditions. After the precipitate had been removed, 0.2 ml of the clear orange supernatant was added to a reaction mixture consisting of 16.0 mg IgG, 0.8 ml DMF and 4.0 ml of PBS. The mixture was stirred for 4 hr in the dark at 4°C and centrifuged at 9,500 x g for 20 min. Free MTX was

separated from the MTX-IgG conjugate by column chromatography on Biogel P100 and then by dialysis overnight against 1 litre of PBS. The average incorporation of MTX was 5 to 6 moles of MTX per mole of IgG. Repeat chromatography of the conjugates after they had been stored for 1 week at 4°C and pH 7.1 gave a single peak of radioactivity that eluted at the position of the conjugates in the initial chromatography. This result shows that there was no dissociation of label from MTX or of the labeled MTX from the conjugate.

K. SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS

Slab-gel polyacrylamide gel electrophoresis (PAGE) was carried out essentially according to Laemmli (1970) with a BioRad vertical slab gel apparatus, model 220. Slab gels were composed of a stacking gel of 3% (w/v) acrylamide, height 2.5 cm, and a running gel of 7.5% (w/v) acrylamide, height 8.5 cm. The gels were 1.5 mm in thickness. The electrophoresis buffer was 25 mM Tris, pH 8.6, containing 192 mM glycine and 0.1% (w/v) SDS. Electrophoresis was carried out with tap water (approximately 15°C) circulated through the inner chamber as a coolant. A constant current of 40 mA was applied per two slab gels until the samples containing bromophenol blue tracking dye reached the running gel. The current was then increased to 80 mA and electrophoresis was continued for about 2 hr. The gels were then fixed and stained for protein with Coomassie Blue.

L. IN VITRO UPTAKE STUDIES

(i) UPTAKE OF MTX AND ITS CONJUGATES BY EL4 CELLS

Female C57BL/6J mice (25-30g) were inoculated i.p with 10^7 EL4

cells. On the 7th day after inoculation when the ascites fluid rarely contains red blood cells, (Guclu, 1975), the ascites fluid containing EL4 cells was harvested and the tumor cells were washed three times with RPMI 1640 medium without serum at 4°C. In 35 x 10 mm tissue culture dishes 10^7 EL4 cells/ml were incubated with 10 μM [^{3}H]MTX (either free or conjugated to NRG or AELG) in a final volume of 2 ml RPMI 1640 for various times at 37°C and parallel aliquots of cells were incubated at 0°C, a temperature at which uptake of MTX is reported not to take place (Goldman et al., 1968; Henderson and Zevely, 1980). Also, capping and endocytosis of cell-surface-bound antibody do not take place at 0°C (Guclu et al., 1978; Unanue et al., 1973). For determination of Km and Vmax values for MTX uptake, the cells were incubated with 2 ml of MTX solution of concentrations between 0.1 μM and 10 μM for 3 min at 37°C. At the end of incubation, the cells were collected by centrifugation at 600 x g for 3 min and were processed for determination of cell-associated radioactivity as described below.

(iii) EFFLUX OF FREE MTX FROM EL4 CELLS

EL4 cells (10^7/ml) were incubated for 1 hr in serum-free RPMI 1640 with either 1 μM [^{3}H]MTX (1.0 Ci/mmol) or 10 μM [^{3}H]MTX (0.16 Ci/mmol) in 35 x 10 mm tissue culture dishes at 37°C. The cells were then washed 6 times with 10 ml of cold PBS, after which they were incubated at 37°C in 2 ml of medium without MTX. At various times, the cells were washed once with 10 ml of cold PBS then processed for determination of cell-associated radioactivity as described below.

(iii) UPTAKE OF MTX AND ITS CONJUGATES BY M21 CELLS

For determination of the kinetics of uptake of free and conjugated MTX by M21 cells, single cell suspensions were plated at a concentration of 10^6 cells per 35 x 10 mm tissue culture dish. At 72 hr, the cells were washed 3 times with 1 ml of RPMI 1640 medium without serum at 4°C and then 1 ml of 10 μM [^{3}H]MTX, free or conjugated, was added and allowed to incubate at 37°C and 0°C. For obtaining a larger quantity of cells, the initial plating was 3 x 10^6cells in 60 x 15 mm dishes, initial washing was with 3 ml of medium and incubation was with 3 ml of medium containing 10 μM [^{3}H]MTX conjugate. At various times, cells were harvested and processed for determination of cell-associated radioactivity as described below.

For determination of Km and Vmax values for MTX uptake, 1 ml of MTX solution of concentrations between 0.1 μM and 10 μM was added to the dish and incubated for 3 min at 37°C. For determining the effect of the free carrier on uptake of conjugate, M21 cells (3 x 10^6 per 60 x 15 mm dish) were washed at 72 hr with RPMI 1640 without serum and incubated with 3 ml of 10 μM [^{3}H]MTX-AHMG$_R$ together with a ten fold molar excess of either unlabeled MTX, free AHMG$_R$ or MTX mixed with AHMG$_R$. They were processed for the determination of cell-associated radioactivity as described below.

(iv) EFFLUX OF MTX AND CATABOLITES OF MTX-AHMG$_R$ CONJUGATE FROM M21 CELLS

For efflux studies with free MTX, 10^6 washed cells per dish were loaded by incubation for 1 hr at 37°C with MTX at a concentration of 1 or 10 μM. At the end of the incubation period, the cells were washed

10 times with 5 ml of cold PBS after which they were allowed to reincubate in 2 ml of medium without serum at 37°C. For efflux studies with MTX-AHMG$_R$, single cell suspensions were plated in 60 x 15 mm tissue culture dish at a concentration of 3 x 10^6 cells per dish. At 72 hr, the cells were washed 3 times with 3 ml of RPMI 1640 without serum at 4°C, then 3 ml of 10 μM [^{3}H]MTX-AHMG$_R$ was added and incubated at 37°C for 7.5 hr. After incubation, the cells were washed 5 times with 10 ml of cold PBS, then 3 ml of RPMI 1640 medium without serum was added and the cells reincubated at 37°C. In a parallel efflux experiment, 100 μM chloroquine or leupeptin was present in the medium throughout the period of efflux. At the indicated times, the cells were harvested and cell-associated radioactivity determined as described below.

(v) DETERMINATION OF CELL-ASSOCIATED RADIOACTIVITY

At the end of incubation, the cells were washed six times (once for efflux experiments) with 10 ml of cold PBS by centrifuging them at 600 x g for 3 min and then dissolved as described by Johnson et al., (1978) in 2 ml of 0.01M Tris-HCl, pH 7.4 containing 0.1M NaCl, 0.001M EDTA and 0.5% sodium dodecylsulfate. The cell-associated radioactivity in 1 ml of the lysate to which had been added 10 ml Aquasol-2 was determined in a Beckman Model LS 7000 scintillation counter.

(vi) PROTEIN DETERMINATION

The protein content of the cell lysate was determined by the method of Lowry et al., (1951), with bovine plasma albumin used as

standard.

(vii) CALCULATION OF ENDOCYTOSIS OF CONJUGATES

For accurate determination of endocytosed antibody, the cell surface-associated immunoglobulin must be subtracted from the total cell-associated immunoglobulin. This requires that aliquots of cells be incubated at 0-4°C with the antibody preparation and subsequently processed at this temperature for analysis of cell surface-associated immunoglobulin (Willingham and Pastan, 1983). At higher temperatures endocytosis and catabolic elution of bound antibody could take place (Willingham and Pastan, 1983). If the cell-surface-associated radioactivity measured for each specific incubation time at 0°C were assumed to be the amount not endocytosed at 37°C, the net uptake would be calculated by subtracting each 0°C value from the corresponding 37°C value. However, a more precise determination of the amount endocytosed at 37°C may be obtained by subtracting the amount of antibody conjugate that is bound at the beginning of incubation at 37°C; 15 pmol/mg protein for MTX-AELG, 30 pmol/mg protein for MTX-AHMG$_{R+T}$, 40 pmol/mg protein for MTX-AHMG$_R$ and 80 pmol/mg protein for MTX-MAB. Results of both calculations are presented.

(viii) CATABOLISM OF CONJUGATES BY M21 CELLS

Cells, preloaded with conjugate for 7.5 hr as above, were allowed to efflux at 37°C for 9.5 hr, then separated from the incubation medium by centrifugation. A homogenate was made by first exposing cells to 40 mM KCl containing 10 mM EDTA pH 7.0, then disrupting them

in a Dounce homogenizer with a tight-fitting pestle (80 strokes). The homogenate was centrifuged at 9,500 x g for 20 min and 1 ml of the supernatant was passed through a column (1 x 20 cm) of Biogel P100. The incubation medium used for efflux was lyophilized and taken up in 10 ml of PBS. After centrifuging at 9,500 x g for 20 min, 1 ml of the supernatant was passed through a column (1 x 20 cm) of Biogel P100. In both cases fractions of 1 ml were collected and counted for radioactivity.

(ix) DIHYDROFOLATE REDUCTASE (DHFR) ASSAY

The assay method of Peterson et al., (1975b) was used to determine inhibition of DHFR by MTX and the low molecular weight radioactivity-containing fractions from the cell extract and efflux medium. Reaction mixtures contained 0.5M sodium acetate buffer, pH 6.0, 0.6 M KCl, 50 μM NADPH, 33 mM dihydrofolate and 0-15 pmole of MTX or corresponding amounts of test fractions in a total volume of 3 ml.

(x) IN VITRO INHIBITION OF GROWTH OF EL4 AND M21 CELLS

EL4 cells obtained from the ascites fluid of C57BL/6J mice were kept in culture for 48 hr in RPMI 1640 medium supplemented with 10% fetal calf serum. At 48 hr, the cells were plated at a concentration of 0.5×10^5 cells per ml in 35 x 10 mm tissue culture dishes. M21 cells from in vitro passage were similarly plated in 35 x 10 mm tissue culture dishes at a concentration of 0.5×10^5 cells per ml in RPMI 1640 medium supplemented with 10% fetal calf serum. MTX in the free or conjugated forms at various concentrations was added to the EL4 and M21 cells 24 hr after plating and incubated for a further 72 hr, after

which they were harvested and counted in triplicate in a Coulter counter to determine the inhibition of growth. The IC_{50} is the concentration for 50% inhibition of growth.

M. IN VIVO UPTAKE STUDIES

(i) UPTAKE OF MTX AND ITS CONJUGATES BY TUMOR AND NORMAL TISSUES IN EL4 LYMPHOMA-BEARING MICE

Female C57BL/6J mice were inoculated i.p. with 10^7 EL4 cells. On the 6th day postinoculation mice were injected i.p. with 5 mg/kg of [^{3}H]MTX either free, conjugated to AELG or conjugated to NRG, in a total volume of 1 ml of PBS. At various times thereafter tumors were drained from the peritoneal cavity with a needle, the animals were killed by decapitation, and their blood was collected and tissues removed. Tumor cells were separated from ascites fluid by centrifugation at 600 x g for 3 min and, after being washed 5 times with 15 ml of PBS, were dried to constant weight at 60°C. The ascites fluid was air-dried in Spectrapor dialysis tubing. Collected blood was allowed to clot at 37°C, and then centrifuged; the resulting serum was air-dried. Tissues were dried to constant weight at 60°C.

For counting of radioactivity, samples were oxidized in an Oxymat sample oxidizer. The isotopic recovery after oxidation was >95% with a memory of <0.05%. Blanks were oxidized between samples to eliminate this memory effect. ^{3}H was counted as ^3H_2O in an Oxifluor-H_2O scintillation cocktail in a Beckman LS 7000 scintillation counter with an efficiency of 37.5%.

(ii) UPTAKE OF MTX AND ITS CONJUGATES BY TUMOR AND NORMAL TISSUES IN HUMAN MELANOMA M21 BEARING NUDE MICE

Female BALB/c nude mice (20-25g) were inoculated subcutaneously with 2 x 10^6 M21 cells in the right flank. Three weeks postinoculation when the tumor volume averaged 874 mm^3, the mice were injected i.v. through the tail vein with 5 mg/kg of [^{3}H]MTX either free, conjugated to $AHMG_{R+T}$ or conjugated to NRG in a total volume of 0.4 ml PBS. At various times thereafter, the mice were killed by decapitation, their blood was collected, and tissues were removed. The tissues were dried to constant weight at 60°C prior to oxidation in the Oxymat sample oxidizer. Collected blood was allowed to clot at 37°C and oxidized.

Fifty microlitre aliquots of sera from mice killed at the different time intervals were precipitated with 950 µl of 10% TCA and after centrifugation, the supernatant and precipitate were counted after addition of 10 ml of Aquasol-2 scintillation cocktail.

N. PREPARATION OF HOMOGENATES OF LIVER AND EL4 CELLS

Freshly removed liver (2.2 g, wet weight) from an adult female C57BL/6J mouse was homogenized in 10 ml of 0.1M sodium acetate buffer pH 4.6, using a Potter-Elvehjem homogenizer (10 passes at 2,000 rpm). An EL4 cell homogenate was obtained by homogenizing 10^8 EL4 cells under the same conditions as for the mouse liver except that 80 passes at 2,000 rpm were made. A typical protein concentration in the homogenate was 7 to 8 mg/ml.

O. DETERMINATION AND CHARACTERIZATION OF HYDROLYTIC ACTIVITY OF LIVER HOMOGENATES ON THE MTX-NRG CONJUGATE.

For determination of the hydrolytic activity of liver homogenates, a typical reaction mixture consisted of: 0.5 ml MTX-NRG conjugate, 0.5 ml liver homogenate, and 1.2 ml of 0.1 M acetate buffer pH 4.6 in a total volume of 2.2 ml.

To determine the effect of various agents on the hydrolytic activity, reaction mixtures contained one of the following: 9.4×10^{-5} M antipain, 8.6×10^{-4}M N α-p-tosyl-L-lysine chloromethyl ketone, 0.64×10^{-3}M EDTA, 0.64×10^{-3} M DTT, 9.2×10^{-5} M leupeptin, 9.2×10^{-5} M pepstatin, 0.64×10^{-3} M calcium chloride and 0.64×10^{-3} M iodoacetate. The reaction mixtures were incubated at 37°C for 12 to 28 hr. At the end of the incubation, the mixtures were centrifuged at 10,000 x g for 20 min and 1 ml of the supernatant was passed through a Biogel P100 column (1 x 20 cm) and eluted with 0.1M acetate buffer pH 4.6. Fractions of 1 ml were collected and counted for radioactivity.

P. HYDROLYSIS OF THE MTX-NRG CONJUGATE BY EL4 CELL HOMOGENATES

The incubation conditions and assay for radioactivity were the same as that for liver homogenates above.

Q DEAE-CELLULOSE CHROMATOGRAPHY

The low molecular weight fragment obtained from the efflux medium of M21 cells previously incubated with MTX-AHMG$_R$ and from the MTX-NRG conjugate incubated with liver homogenate was further purified by adsorbing on a column (1.2 x 30 cm) of DEAE-cellulose equilibrated with 5 mM phosphate buffer pH 7.0 and eluted with a linear gradient of

1 litre of 5 mM phosphate buffer in the mixing chamber and 1 litre of 0.5 M NaCl in the same buffer in the reservoir. Fractions of 19 ml were collected (Baugh et al., 1973).

R. THIN LAYER CHROMATOGRAPHY

Thin layer chromatographic analysis was performed on cellulose-coated glass plates with 0.1M glycine containing 2% EDTA pH 9.0 as the solvent system (Silber et al., 1963).

RESULTS

A. PROPERTIES OF THE MTX-ANTIBODY CONJUGATES

(i) MOLAR INCORPORATION AND RETENTION OF DRUG ACTIVITY

An average of 5 to 6 moles of MTX per mole of IgG was usually obtained after conjugation. This incorporation ratio ensures retention of antibody activity. The retention of MTX activity in conjugates was determined by comparing the inhibition of DHFR by equimolar amounts of MTX either free or incorporated in conjugates. This comparison always showed that approximately twice the amount of conjugated MTX was necessary for 50% inhibition of enzyme activity (Table 2, page 119).

(ii) RETENTION OF ANTIBODY ACTIVITY

Serial dilution assay of 2×10^7 EL4 cells/ml starting with 1 mg/ml of MTX-AELG, failed to reveal any decrease in membrane immunofluorescence titre (25 µg/ml) compared to the parent AELG. A similar dilution assay of 10^6 melanoma cells/ml, starting with 1 mg/ml of MTX-antibody preparation gave titres of 1.56, 12.5 and 25 µg/ml for MTX-MAB, MTX-$AHMG_R$ and MTX-$AHMG_{R+T}$ respectively. A comparable immunofluorescence titre was observed for the respective unconjugated antibody preparations, confirming that the conjugated antibody still retains essentially its full capacity to bind to its antigen.

Conjugation was done with IgG obtained by precipitation at 40% saturation with $(NH_4)_2SO_4$ followed by re-precipitation at 33% saturation. Its purity was superior to IgG precipitated only with 40% saturated $(NH_4)_2SO_4$ and comparable to IgG purified by affinity chromatography on protein A Sepharose as shown by SDS-PAGE and agar gel immunoelectrophoresis (figure 4).

Figure 4: PURITY OF IgG AS DETERMINED BY AGAR GEL IMMUNOELECTROPHORESIS

Agar gel immunoelectrophoresis of;

A: Upper well - rabbit serum

Lower well - protein A purified rabbit IgG

Centre trough - goat antirabbit whole serum.

B: Upper well - $AHMG_R$ precipitated with 33% $(NH_4)_2SO_4$

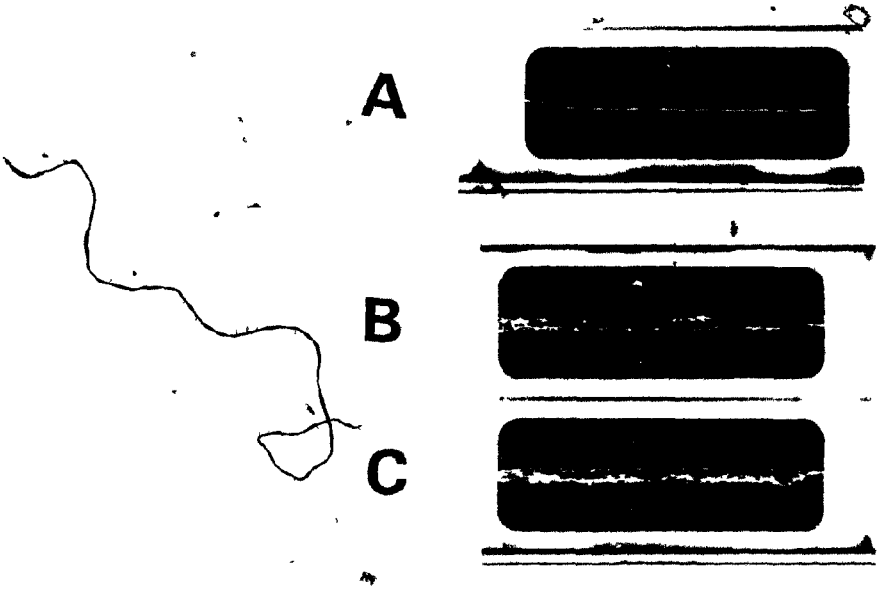
Lower well - MTX-$AMHG_R$ precipitated with 33% $(NH_4)_2SO_4$

Centre trough - goat antirabbit whole serum.

C: Upper well - $AHMG_R$ precipitated with 33% $(NH_4)_2SO_4$

Lower well - MTX-$AHMG_R$ precipitated with 33% $(NH_4)_2SO_4$

Centre trough - goat antirabbit 7S globulin.


A
B
C

B. IN VITRO UPTAKE STUDIES WITH EL4 CELLS

(i) UPTAKE OF FREE MTX

The uptake of free MTX by EL4 cells incubated at 0° and 37°C was carried out with an external MTX concentration of 10 μM. The cell-associated radioactivity of cells incubated with free MTX at 0°C was not different from the background activity after completion of washing. The uptake of free MTX by cells incubated at 37°C was rapid and leveled off after 30 min, at 30 pmol/mg protein (Figure 5). The Eadie-Hofstee plot for the uptake of free MTX at various extracellular concentrations between 0.1 and 10 μM gave a Vmax of 1.8 pmol MTX/min/mg protein and a Km of 6.4 μM (Figure 6).

(ii) UPTAKE OF MTX-NRG

When EL4 cells were incubated at 0°C with [^{3}H]MTX-NRG at an external drug concentration of 10 μM, little tritium was observed to be bound to the cells and there was no progressive increase. However, incubation at 37°C led to a progressive increase in cell-associated MTX (Figure 7), but the rate of uptake of the NRG conjugate gradually decreased so that there was a leveling off at 24 pmol/mg protein at the end of the observation period.

(iii) UPTAKE OF MTX-AELG

In contrast to the cells incubated with MTX-NRG at 0°C, when EL4 cells were incubated with MTX-AELG at the same temperature (i.e. 0°C) there was a progressive increase in binding, which leveled off around 5 hr at approximately 15 pmol MTX/mg protein, suggesting that AELG reached equilibrium with the available binding sites (Figure 7); this

level corresponds to the amount that initially bound to these cells at 37°C, where equilibration would occur much more rapidly. Incubation at 37°C led to a progressive increase in cell-associated MTX with the AELG conjugate and its rate of uptake remained steady at approximately 10 pmol/mg protein per hr for at least 6 hr when the observations ceased because the proportion of trypan blue permeable (i.e. nonviable) cells started to increase.

Figure 7 inset, shows the net uptake of MTX at 37°C calculated by subtracting, from the total amount bound at 37°C at specified times, either the amount bound after 5 hr at 0°C or the amount bound at each time interval. The result shows that at 6 hr cells incubated with MTX-AELG endocytosed more MTX (40 pmol/mg protein) than did cells incubated either with MTX alone (30 pmol/mg protein) or with MTX-NRG.

(iv) EFFLUX OF MTX FROM EL4 CELLS PRELOADED WITH MTX

When MTX was allowed to efflux from MTX-loaded EL4 cells by incubating them in MTX-free medium, the concentration of intracellular MTX declined rapidly, leveling off at 5 pmol per mg protein within 40 min (Figure 9 inset). This measurement of residual firmly-bound MTX provides an estimate of the intracellular level of DHFR based on its high affinity stoichiometric binding of MTX (Sirotnak, 1980).

C. IN VIVO UPTAKE STUDIES IN EL4 LYMPHOMA BEARING MICE

Figure 8 shows how free MTX, MTX-AELG and MTX-NRG were distributed at various times after administration, in EL4 cells, liver, lungs, kidneys, brain, spleen, serum and ascites fluid of tumor-bearing mice.

Figure 5: UPTAKE OF FREE [^{3}H] MTX BY EL4 LYMPHOMA CELLS.

Cells (10^7 per culture dish) were incubated at 37°C with 10 μM MTX in a total volume of 2 ml. At the times indicated, cells were washed six times with 10 ml PBS at 0°C. The intracellular MTX, determined after the cells were dissolved in SDS-containing buffer, reached a constant level of 30 pmol/mg protein within the 1st hr. 1 mg of protein is equivalent to 1.73×10^7 cells. Each point represents the mean of three determinations.

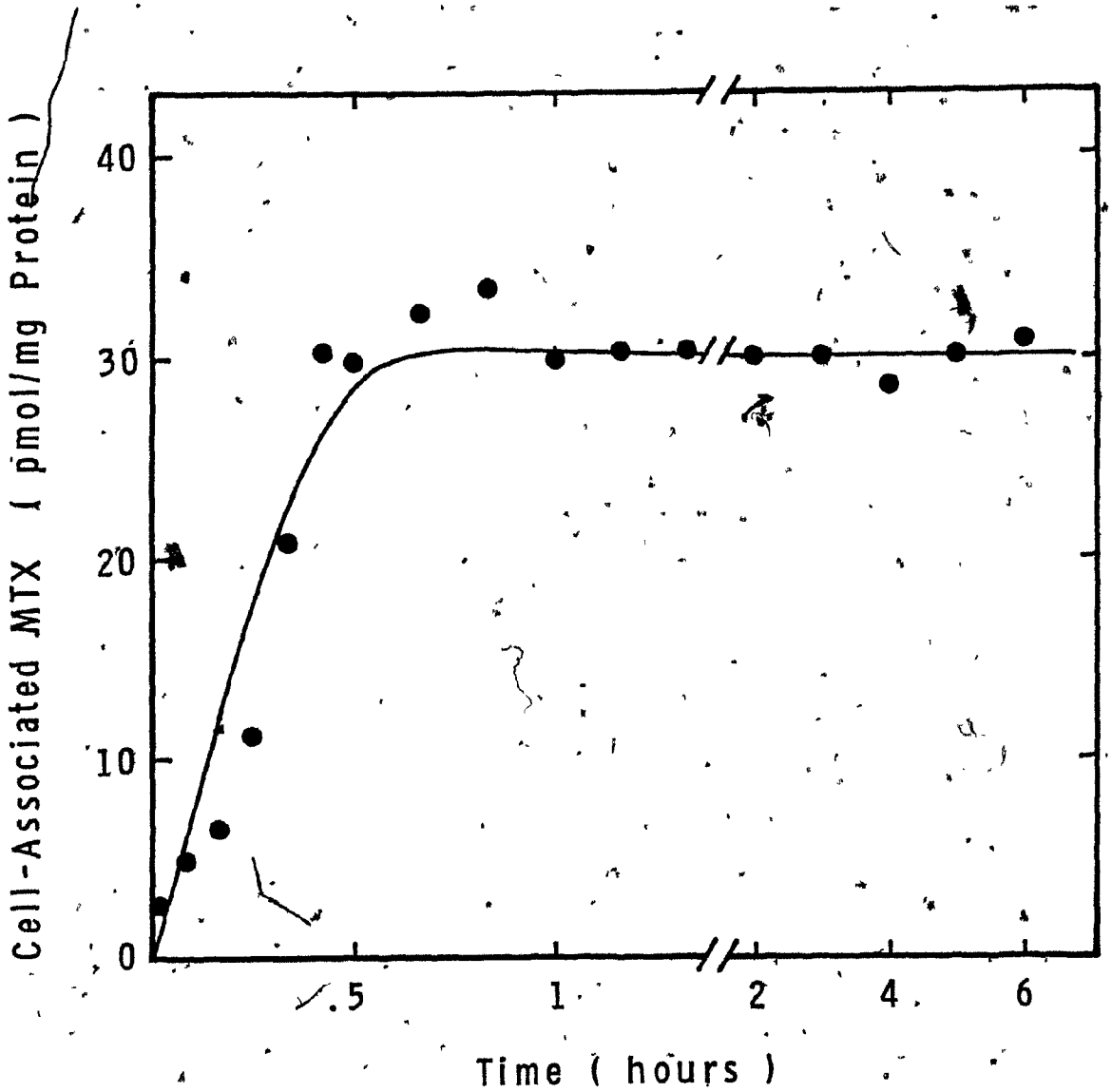

Cell-Associated MTX (pmol/mg Protein)
40
30
20
10
0
.5
1
2
4
6
Time (hours)

Figure 6: UPTAKE OF MTX AT VARIOUS CONCENTRATIONS BY EL4 LYMPHOMA CELLS

Cells (10^7 per culture dish) were incubated at 37°C for 3 min with [^{3}H]MTX at the concentrations indicated in a total volume of 2 ml. After incubation, cells were washed, were dissolved in SDS-containing buffer and their radioactivity were measured as described in Materials and Methods. Inset: Eadie-Hofstee plot to obtain the maximal rate of uptake (Vmax) and the MTX concentration giving half maximal uptake (Km).

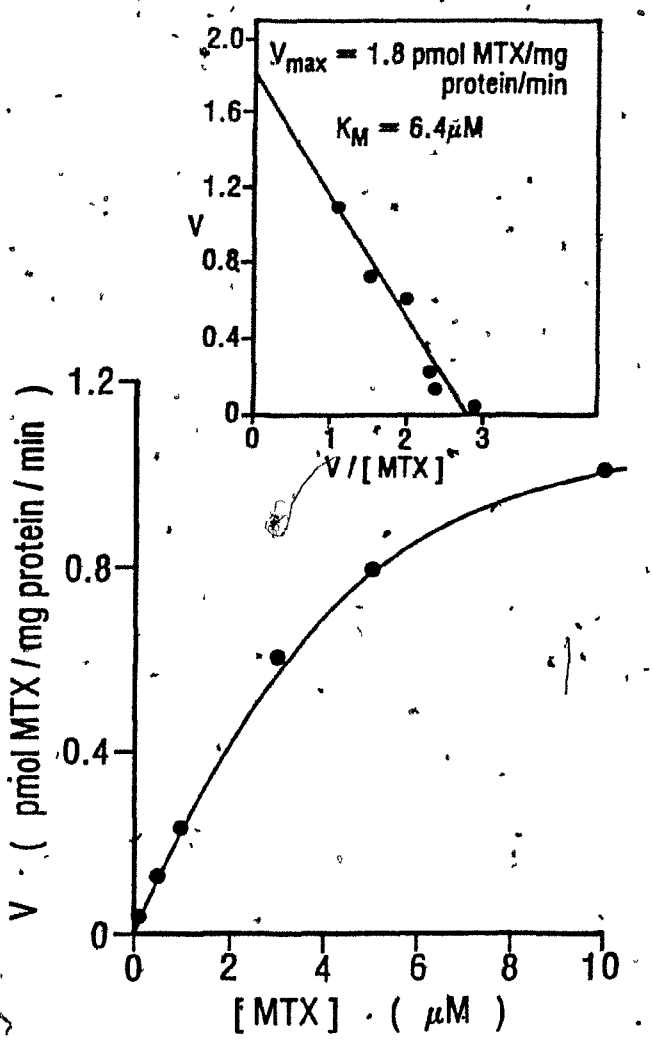
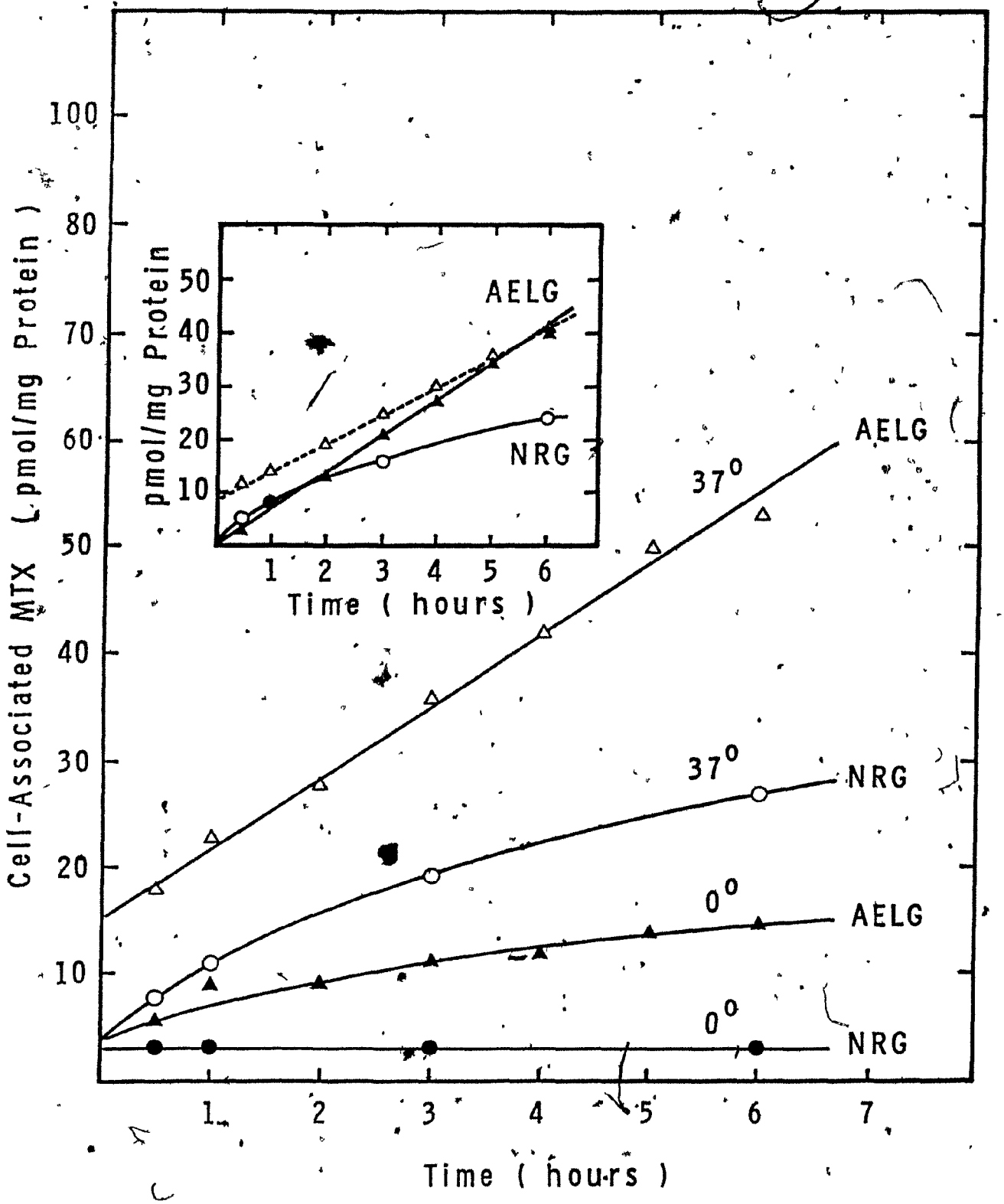

V_{max} = 1.8 pmol MTX/mg protein/min
K_M = 6.4μM
V
V / [MTX]
V · (pmol MTX / mg protein / min)
[MTX] · (μM)

Figure 7: UPTAKE OF [^{3}H]MTX-AELG AND [^{3}H]MTX-NRG BY L 4 LYMPHOMA CELLS

Cells (10^7 per culture dish) were incubated at 37°C and 0°C with [^{3}H]MTX-AELG or [^{3}H]MTX-NRG to give a concentration of 10 μM MTX in a total volume of 2 ml. At the times indicated cells were washed six times with 10 ml PBS at 0°C. The intracellular MTX was determined after dissolving the cells in SDS-containing buffer. Each point represents the mean of three determinations.

Inset: Net uptake of MTX at 37°C calculated by subtracting, from the total amount bound at 37°C at specified times, either the amount bound after 5 hr at 0°C (solid lines) or the amount bound at each time interval (broken line). ▲ and Δ, AELG; O, NRG.


Cell-Associated MTX (pmol/mg Protein)
100
80
70
60
50
40
30
20
10
1
2
3
4
5
6
7
Time (hours)
37°
AELG
37°
NRG
0°
AELG
0°
NRG
pmol/mg Protein
50
40
30
20
10
AELG
NRG
1
2
3
4
5
6
Time (hours)

(i) TISSUE DISTRIBUTION OF FREE MTX

The uptake of MTX reached peak levels in all tissues including serum at between 30 and 60 min. Peak levels were greatest in liver and kidney (18-20% of administered radioactivity per g dry weight). Lung, spleen and tumor took up lesser amounts (8.5, 5.5 and 4% respectively) and the lowest uptake was in brain (0.25%). Tumor cells retained a substantial portion of the MTX taken up; approximately 45% of the peak value remained at 24 hr. MTX in ascites fluid declined steadily, reaching 0.8% of the administered dose per ml at 3 hr and 0.01% at 24 hr. After reaching its peak at 1 hr, the level in serum declined to 0.3% per ml at 3 hr and [illegible]03% per ml at 24 hr. In liver, spleen, kidney and lungs, the major part of the accumulated MTX was cleared by 3 hr and 1% or less remained at 24 hr. Although the peak level of uptake of MTX in brain was much lower than in tumor cells, its rate of clearance (judged by comparing radioactivities expressed as percentages of the peak activities) was slower than from any other normal tissue and was similar to that from EL4 cells, declining from 0.25% of the administered dose per g dry weight to 0.12% by 3 hr and remaining at that level at 24 hr.

(ii) TISSUE DISTRIBUTION OF MTX-AELG AND MTX-NRG CONJUGATES

The most striking finding was the difference in uptake between MTX-AELG and MTX-NRG by tumor cells. The amount of radioactivity in tumor cells from animals given the AELG conjugate increased progressively until 3 hr after administration, those cells contained 15% of the administered dose per g dry weight. This level was

sustained for the rest of the 24 hr observation period. In contrast, the uptake of the MTX-NRG conjugate leveled off, at 1 hr, at 6% of the administered dose per g dry weight. When the uptake of MTX-AELG, MTX-NRG and free MTX were compared 3 hr after administration (i.e. after reaching plateau levels), the level of MTX administered as the AELG conjugate was 2.5 times that of MTX administered as the NRG conjugate and 6 times that of MTX administered free.

In ascites fluid substantial levels of both conjugates persisted, in contrast to the rapid clearance of free MTX (7-9% of the administered dose per ml compared to 0.01% at 24 hr for free MTX). Up to 3 hr, the proportion of the administered dose of MTX-AELG that remained in ascites fluid was slightly greater than that of MTX-NRG. Serum levels of both conjugates rose rapidly during the first hour, as did that of free MTX. As was true of the ascites-fluid levels, throughout the 24 hr period of observations, the serum levels of mice that received one of the conjugates were higher than those of mice that received free MTX.

In contrast to the difference in the uptake of MTX-AELG and MTX-NRG by tumor cells, the pattern of uptake of the two conjugates was similar in all the other tissues studied, with the possible exception of brain. Brains of mice given MTX-AELG, showed a somewhat higher MTX uptake at 24 hr than those of mice given MTX-NRG, but this tissue took up much lower levels of MTX, free or conjugated, than any other tissue analyzed. The rate of uptake of conjugated MTX by liver, kidney and lungs was slower than that of free MTX, and the extent of

uptake was less than the peak value attained by free MTX at 1 hr. In all tissues, conjugated MTX persisted throughout the 24 hr observation period, unlike free MTX which was cleared very quickly (except perhaps in brain). In spleen and brain, the extent of uptake of conjugates exceeded that of free MTX and these higher levels also persisted.

When the results of uptake of the three agents were expressed as pmol of MTX per mg of EL4 cell protein, it is obvious that all uptake levels exceeded intracellular DHFR (Figure 9).

D. CATABOLISM OF MTX-NRG CONJUGATE BY EL4 CELL HOMOGENATE

To determine the ability of EL4 lymphoma cells to catabolize MTX-IgG conjugates, MTX-NRG was incubated with a homogenate of EL4 cells at pH 4.6 to favour lysosomal activity. Biogel P100 chromatography of the incubation mixture then showed the appearance of a second radioactive peak eluting at a position slightly ahead of that for free [^{3}H]MTX (Figure 10).

E. IN VITRO UPTAKE STUDIES WITH HUMAN MELANOMA M21 CELLS

(i) UPTAKE OF FREE MTX

The uptake of free MTX by M21 cells incubated at 0° and 37°C was measured using an external MTX concentration of 10 μM. As with the EL4 cells, the cell-associated radioactivity of M21 cells incubated with free MTX at 0°C was not different from the background activity after completion of washing. The uptake of free MTX by cells incubated at 37°C was also rapid, reaching a plateau within 60 min of approximately 16 pmol/mg protein (Figure 11B). The Eadie-Hofstee plot for the uptake of free MTX at various extracellular concentrations

Figure 8: UPTAKE AND CLEARANCE OF FREE MTX, MTX-AELG AND MTX-NRG IN ASCITES FLUID, SERUM AND TISSUES OF EL4-LYMPHOMA-BEARING MICE

Mice were inoculated i.p. with 10^7 EL4 cells and, 6 days later, were injected i.p. with a 5 mg/kg dose of either free [^{3}H]MTX (●), [^{3}H]MTX-AELG (▲), or [^{3}H]MTX-NRG (Δ), in a total volume of 1 ml of PBS. At the times indicated, tissue samples were obtained and were counted for ^{3}H radioactivity as described in Materials and Methods. Tissue-associated radioactivity is expressed as the precentage of the administered dose per g dry weight of tissue (%/g) or from ascites fluid and serum per ml (%/ml). Each value is the mean of - determinations on 3 samples from different animals.

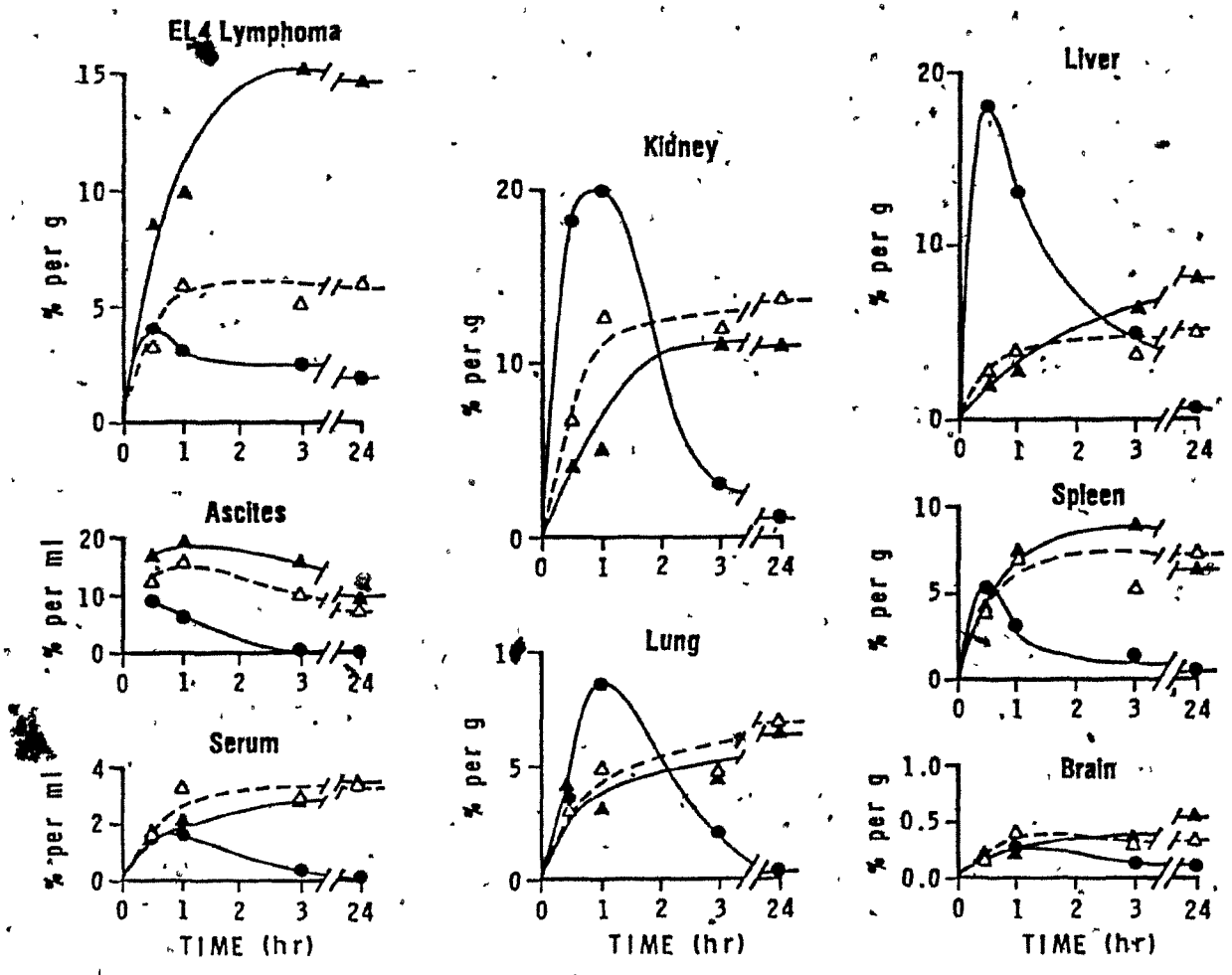

EL4 Lymphoma
% per g
Ascites
% per ml
Serum
% per ml
TIME (hr)
Kidney
% per g
Lung
% per g
TIME (hr)
Liver
% per g
Spleen
% per g
Brain
% per g
TIME (hr)

Figure 9: UPTAKE OF FREE MTX (●), MTX-AELG (▲) AND MTX-NRG (△) BY TUMOR CELLS IN EL4-LYMPHOMA-BEARING MICE.

The experimental conditions were as specified in Figure 8 but the results of determination of cell-associated radioactivity are expressed as pmol of MTX per mg protein. The dotted line shows the DHFR level in these cells, estimated from the data for efflux shown in the inset.

Inset, efflux of MTX from EL4 lymphoma cells in vitro. Cells (10^7/ml) in serum-free RPMI medium were incubated for 1 hr with [^{3}H]MTX at a concentration of either 1 μM (●) or 10 μM (▲), after which they were washed and transferred to MTX-free medium. At the indicated times, radioactivity remaining associated with the cells was determined as described in Materials and Methods.

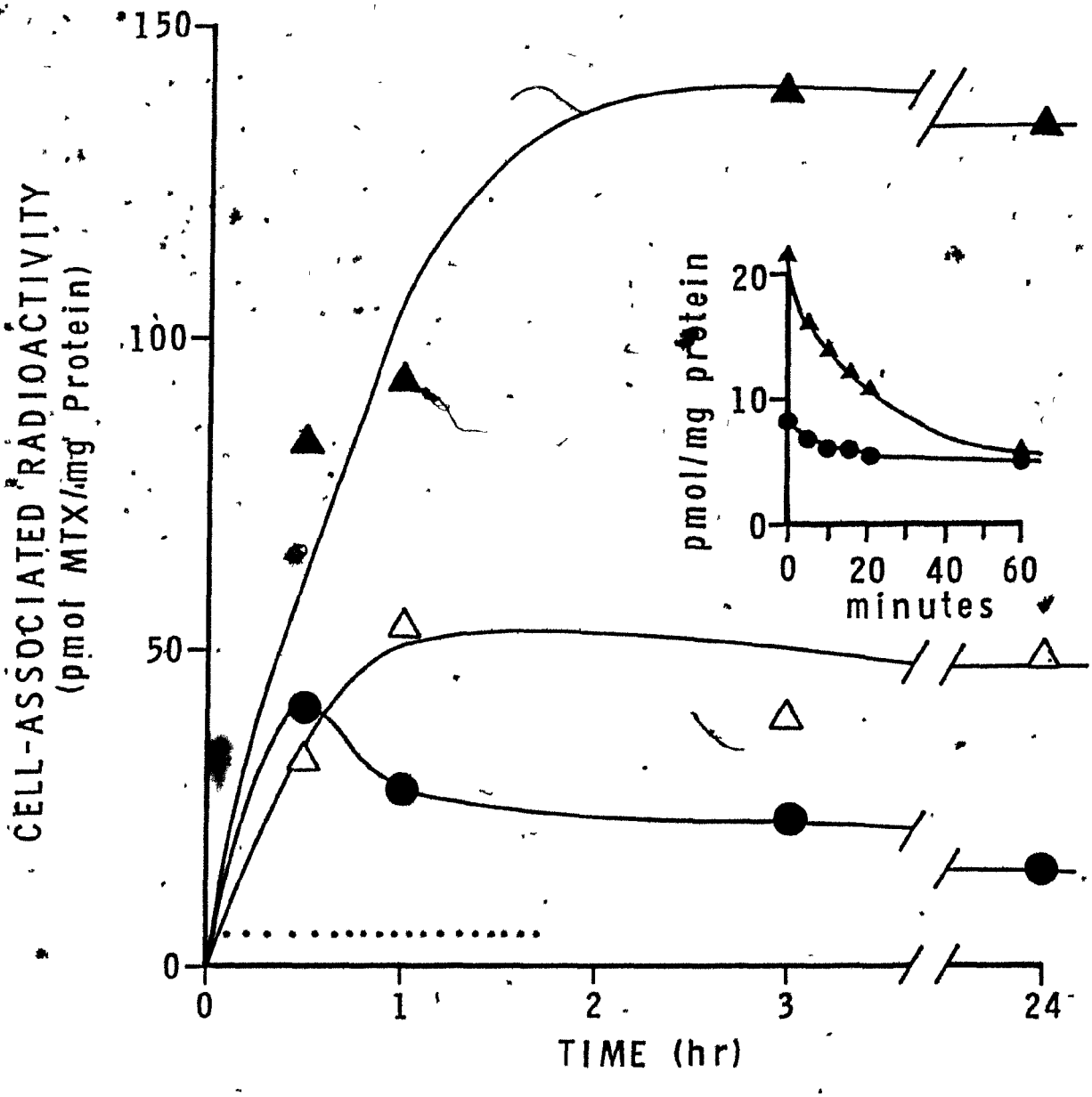
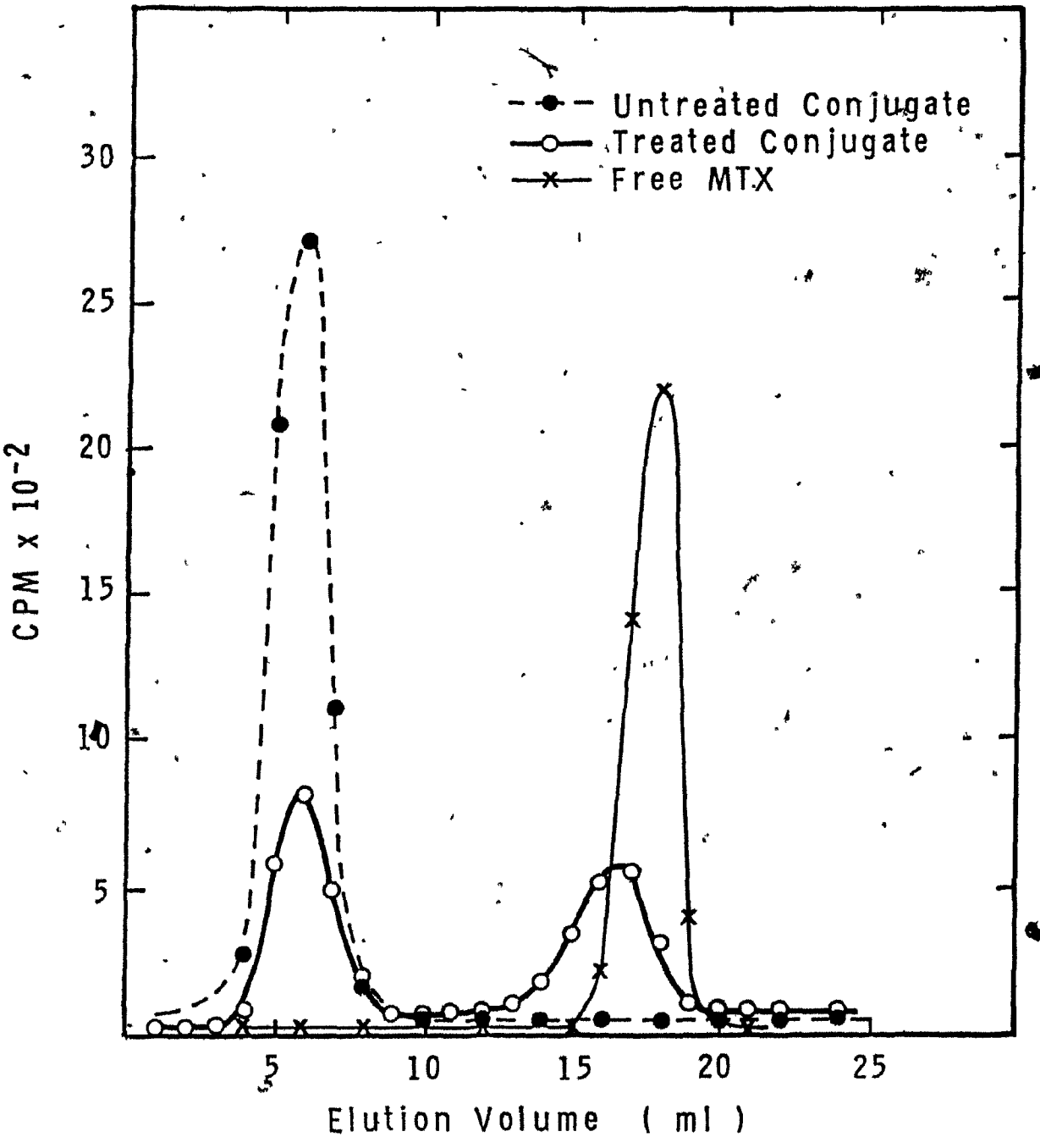

CELL-ASSOCIATED RADIOACTIVITY
(pmol MTX/mg Protein)
150
100
50
0
0
1
2
3
24
TIME (hr)
pmol/mg protein
20
10
0
0
20
40
60
minutes

Figure 10: BIO-GEL P100 ELUTION PROFILE OF [^{3}H]MTX-NRG BEFORE AND AFTER INCUBATION WITH EL4 LYMPHOMA HOMOGENATE

The incubation was carried out for 28 hr with 0.5 ml MTX-NRG conjugate, 0.5 ml liver homogenate, and 1.2 ml of 0.1 M acetate buffer pH 4.6 in a total volume of 2.2 ml. At the end of the incubation, the mixtures were centrifuged at 10,000 x g for 20 min and 1 ml of the supernatant was passed through a Biogel P100 column (1 x 20 cm) and eluted with 0.1 M acetate buffer pH 4.6. Fractions of 1 ml were collected and counted for radioactivity. Note that all ^{3}H-activity of the untreated conjugate emerged in one high-molecular-weight peak, whereas after incubation a second, low-molecular-weight peak emerged slightly ahead of the position for a sample of free [^{3}H]MTX passed through the same column.


Untreated Conjugate
Treated Conjugate
Free MTX
CPM x 10-2
30
25
20
15
10
5
5
10
15
20
25
Elution Volume (ml)

between 0.1 and 10 μM gave a Vmax of 3.3 pmol MTX/min/mg protein and a Km of 5.3 μM (Figure 11A inset).

(ii) UPTAKE OF MTX-NRG

The uptake of MTX-NRG by M21 cells incubated at 0° and 37°C was measured also using an external drug concentration of 10 μM. Initially, little tritium (8-10 pmol MTX/mg protein) bound to the M21 cells irrespective of temperature, followed by a slow progressive increase to 18-20 pmol of MTX per mg protein at 6 hr. At no time was the difference between cell bound activity at 0°C and 37°C more than 5 pmol/mg (Figure 12).

(ii) UPTAKE OF MTX-AHMG$_R$, MTX-AHMG$_{R+T}$ AND MTX-MAB

Using an external drug concentration of 10 μM, the uptake at 0° and 37°C of MTX conjugated to AHMG$_R$, AHMG$_{R+T}$ and MAB was measured (Figure 12). When cells were incubated with MTX-AHMG$_{R+T}$ at 0°C there was a progressive increase in binding starting at 17 pmol/mg, and leveling off around 4 hr at approximately 36 pmol/mg protein. This latter level was slightly above the amount (30 pmol/mg) that initially bound to cells at 37°C. Incubation with MTX-AHMG$_{R+T}$ at 37°C led to a progressive increase in cell-associated MTX, starting at 30 pmol/mg and leveling off at approximately 80 pmol/mg at 6 hr when observations ceased.

When cells were incubated with MTX-AHMG$_R$ at 0°C there was a progressive increase in binding starting at 40 pmol/mg protein, which then appeared to be leveling off after 4 hr. Incubation with MTX-AHMG$_R$ at 37°C also gave an initial value of cell-assoicated MTX

of 40 pmol/mg protein. There was a rapid increase during the next hour followed by a leveling off to approximately 140 pmol/mg protein at 6 hr. The amount of cell-bound radioactivity during incubation with MTX-AHMG$_R$ was higher than with MTX-AHMG$_{R+T}$ at all observation times whether comparison was made from data at 0°C or at 37°C. Both binding at 0°C and uptake of MTX-MAB far exceeded that for the polyclonal antibody conjugates. Initial binding measured at 0°C or 37°C was approximately 85 pmol/mg protein, two-fold greater than MTX-AHMG$_R$. Unlike MTX-AHMG$_R$, uptake of MTX-MAB at 37°C was linear throughout the observation period reaching 540 pmol/mg protein at 6 hr.

Figure 13 shows the net uptake of MTX-MAB, MTX-AHMG$_R$ and MTX-AHMG$_{R+T}$ calculated by subtracting from the total amount bound at 37°C at specified times either the amount bound at one minute at 37°C (right) or the amount bound at each time at 0°C (left). Those calculated by the former procedure showed that net uptake was highest at all times for MTX-MAB followed by MTX-AHMG$_R$ and MTX-AHMG$_{R+T}$, in that order. At 6 hr the ratios of net uptake were 9.4:2:1 for MTX-MAB:MTX-AHMG$_R$:MTX-AHMG$_{R+T}$. The same order of net uptake was evident at 2 hr and beyond when estimation was based on the second procedure.

Incubation of M21 cells with MTX-AHMG$_R$ in the presence of a ten fold excess of AHMG$_R$ decreased ensuing binding by 40% and the initial rate of uptake by 50%. However, in the presence of a ten fold excess of MTX, the initial binding remained the same, the initial rate

of uptake was slightly increased, and uptake continued at a substantial rate after 1 hr in contrast to the control (Figure 14).

(iv) EFFLUX OF MTX FROM M21 CELLS PRELOADED WITH MTX

M21 cells were preloaded by incubation with free MTX, washed and reincubated in drug-free medium. Cell-associated radioactivity after preloading with 10 μM free MTX declined from 16 pmol/mg to approximately 1.8 pmol/mg within 40 min and remained at that level (Figure 15 inset). Cells preloaded with 1 μM MTX also retained almost that same amount at 40 min and 60 min.

(v) EFFLUX OF MTX-CONTAINING FRACTIONS FROM M21 CELLS PRELOADED WITH MTX-AMHG$_R$

M21 cells that had been preloaded to a drug content of 112 pmol/mg protein by incubation with 10 μM MTX conjugated to AHMG$_R$, retained amounts of MTX greatly in excess of its DHFR level (1.8 pmol/mg) after incubation in conjugate free medium, ie, 65 pmol/mg protein remaining at 12 hr (Figure. 15).

F. IN VIVO UPTAKE STUDIES IN HUMAN MELANOMA M21 BEARING NUDE MICE

Figure 16 shows how free MTX, MTX-NRG and MTX-AHMG$_{R+T}$ were distributed at various times after administration, in M21 melanoma cells, liver, lungs, kidneys, brain, spleen, intestine and serum of tumor bearing nude mice.

(i) TISSUE DISTRIBUTION OF FREE MTX

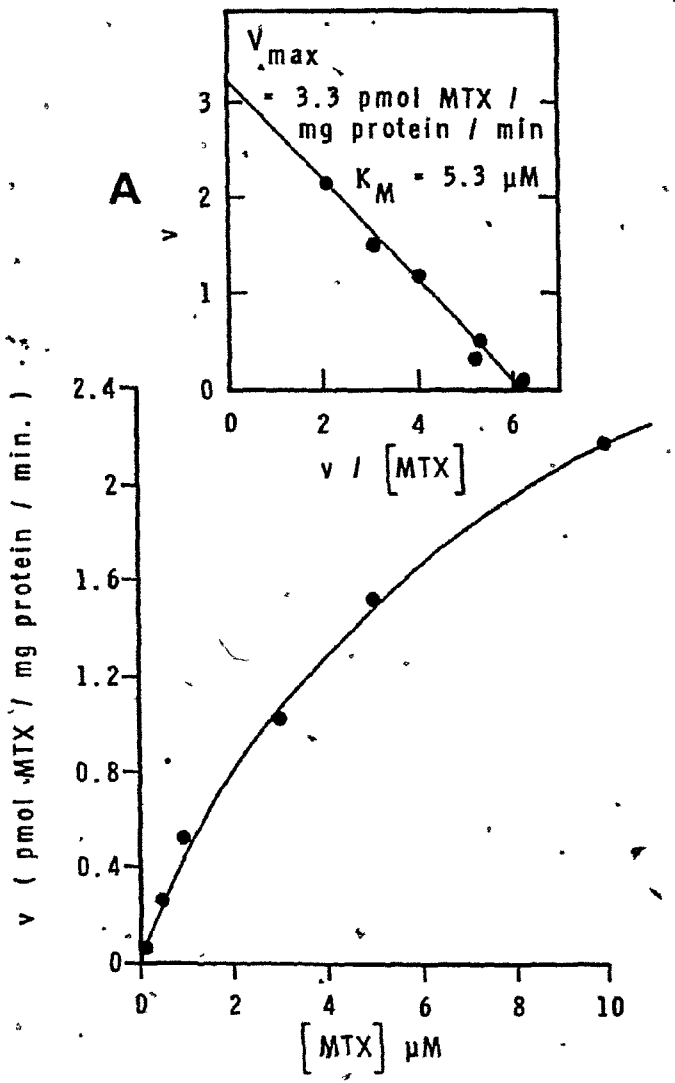
The uptake of MTX peaked in all tissues including serum at 30 min when the tissues were first sampled and declined steadily thereafter. Peak levels were greatest in intestine and liver (15.2% and 8% of the

Figure 11: UPTAKE OF FREE MTX BY M21 CELLS.

11A. Cells (10^6 per culture dish, grown for 72 hr and washed) were incubated in RPMI without serum at 37°C for 3 min with [^{3}H]MTX at the concentrations indicated in a total volume of 1 ml. After incubation, cells were washed, were dissolved in SDS-containing buffer and their radioactivity were measured as described in Materials and Methods.

Inset: Eadie-Hofstee plot to obtain the maximal rate of uptake (Vmax) and the MTX concentration giving half maximal uptake (Km).

11B. Cells (prepared as above) were incubated at 37°C with 10 μM [^{3}H]MTX and intracellular MTX was measured at various times. 1 mg of protein is equivalent to 7.01×10^6 cells.


A
V_{max}
= 3.3 pmol MTX / mg protein / min
K_M = 5.3 µM
v
v / [MTX]
v (pmol MTX / mg protein / min.)
[MTX] µM

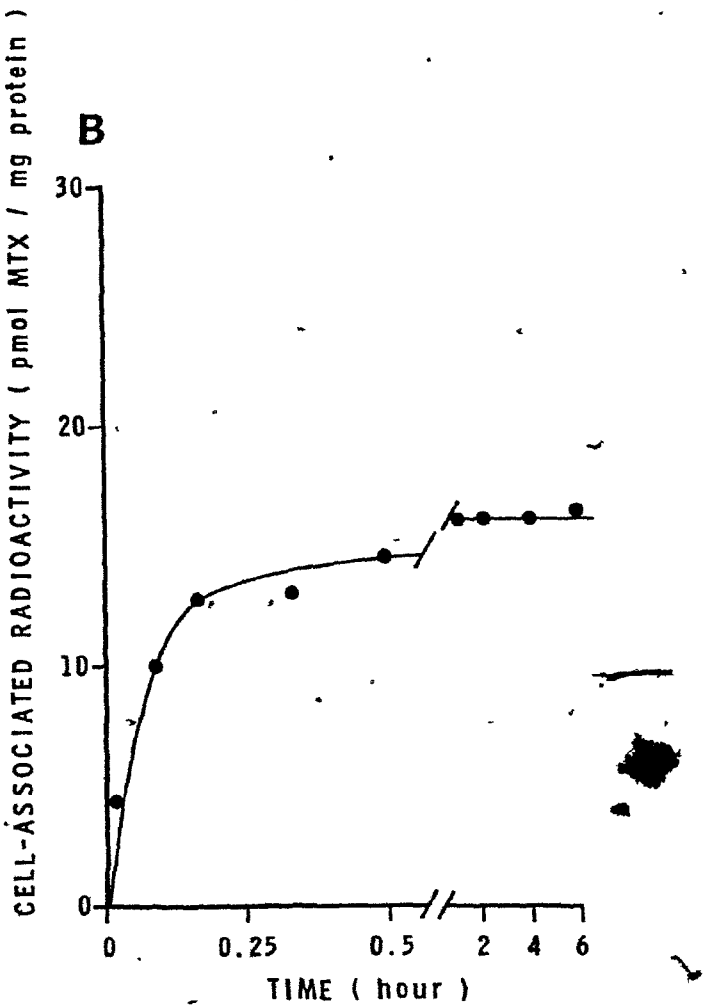

B
CELL-ASSOCIATED RADIOACTIVITY (pmol MTX / mg protein)
TIME (hour)

Figure 12: UPTAKE OF CONJUGATED MTX BY M21 CELLS

Cells (3 x 10^6 per culture dish, grown for 72 hr and washed) were incubated in RPMI without serum at 0°C or 37°C with [^{3}H]MTX-MAB, [^{3}H]MTX-AHMG$_R$, [^{3}H]MTX-AHMG$_{R+T}$ and [^{3}H]MTX-NRG (MTX concentration, 10 µM; total volume 3 ml). At the times indicated, cells were washed, were dissolved in SDS-containing buffer, and cell-associated radioactivity was measured as described in Materials and Methods. Points represent the mean of 3 determinations.

Inset: Results for MTX-MAB plotted to show the complete data.

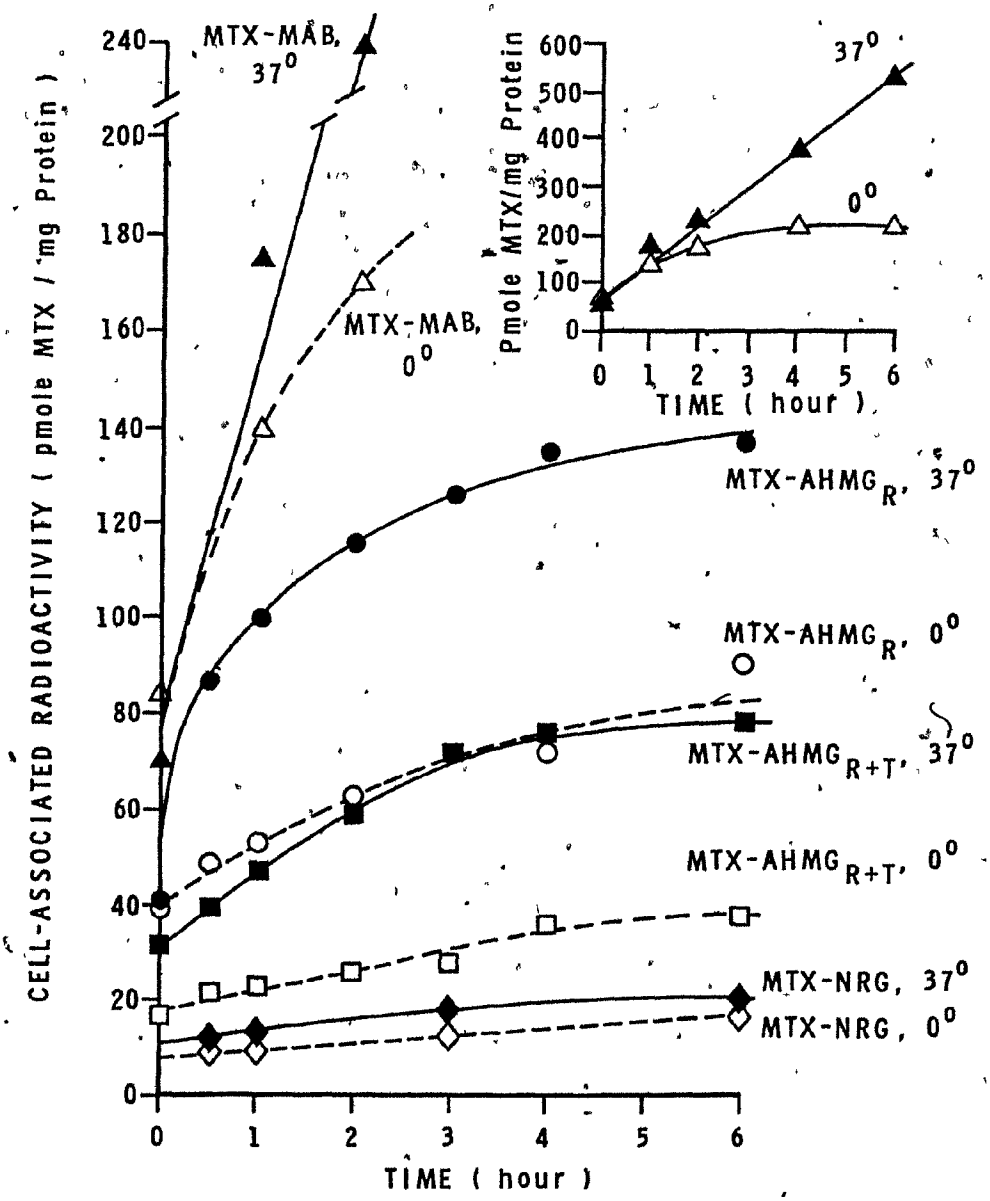

CELL-ASSOCIATED RADIOACTIVITY (pmole MTX / mg Protein)
TIME (hour)
MTX-MAB, 37°
MTX-MAB, 0°
MTX-AHMG$_R$, 37°
MTX-AHMG$_R$, 0°
MTX-AHMG$_{R+T}$, 37°
MTX-AHMG$_{R+T}$, 0°
MTX-NRG, 37°
MTX-NRG, 0°
Pmole MTX/mg Protein
TIME (hour)
37°
0°

Figure 13: Net uptake of MTX-MAB, MTX-AHMG$_R$, and MTX-AHMG$_{R+T}$ calculated by subtracting from the total amount bound at 37°C at specified times either the amount bound at one minute at 37°C (right) or the amount bound at each time at 0°C (left). Data taken from Figure 12.

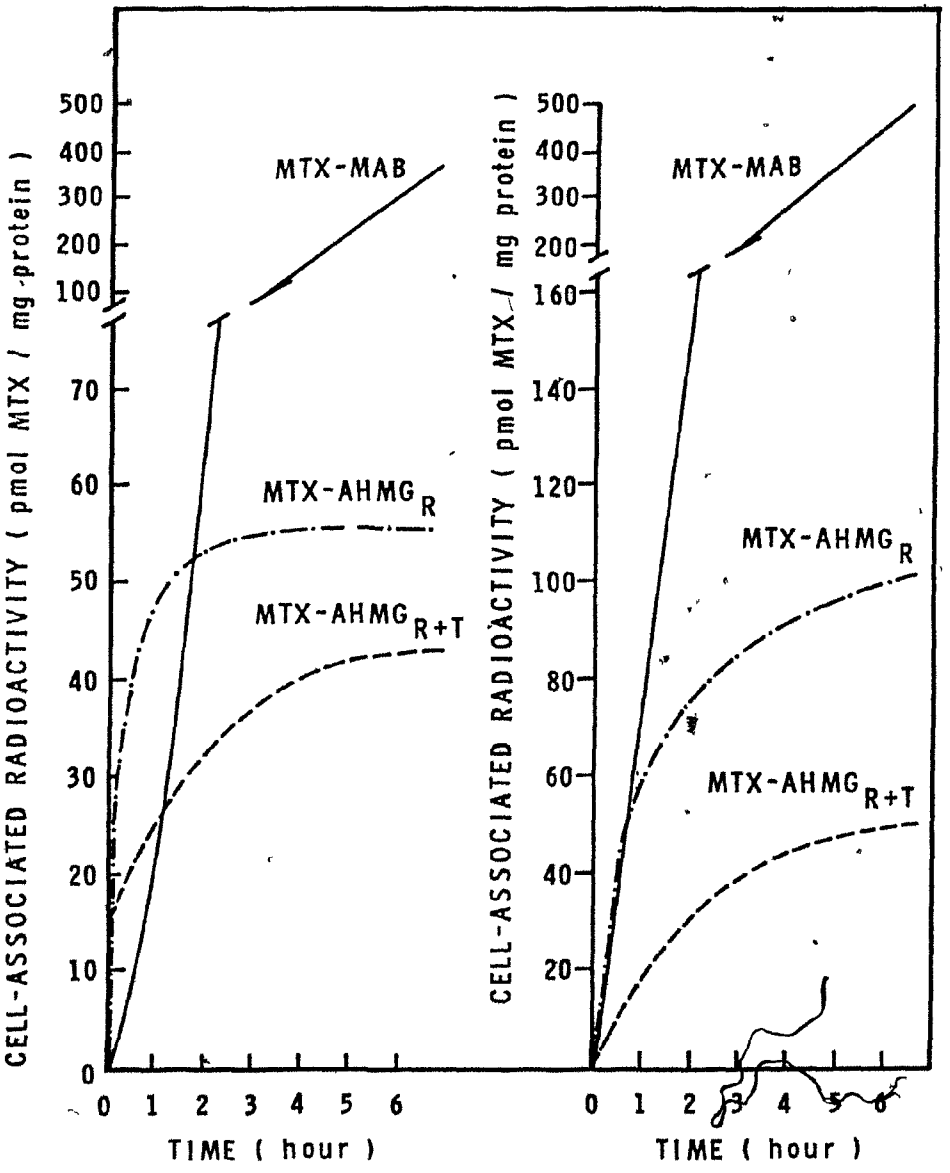

CELL-ASSOCIATED RADIOACTIVITY (pmol MTX / mg protein)
500
400
300
200
100
70
60
50
40
30
20
10
0
MTX-MAB
MTX-AHMG R
MTX-AHMG R+T
0 1 2 3 4 5 6
TIME (hour)
CELL-ASSOCIATED RADIOACTIVITY (pmol MTX / mg protein)
500
400
300
200
160
140
120
100
80
60
40
20
MTX-MAB
MTX-AHMG R
MTX-AHMG R+T
0 1 2 3 4 5 6
TIME (hour)

Figure 14: INHIBITION OF THE UPTAKE OF MTX-AHMG$_R$ BY AHMG$_R$ OR MTX

Cells (3 x 10^6 per culture dish, grown for 72 hr and washed) were incubated in RPMI without serum at 37°C with [^{3}H]MTX-AHMG$_R$ in the presence of one of the following: 100 μM MTX, 3.45 mg AHMG$_R$, 100 μM MTX + 3.45 mg AHMG$_R$ (MTX concentration, 10 μM; total volume 3 ml). At the times indicated, cells were washed, were dissolved in SDS-containing buffer, and cell-associated radioactivity was measured as described in Materials and Methods. Points represent the mean of 3 determinations.

CONTROL, indicates cells incubated with 10 μM MTX-AHMG$_R$.

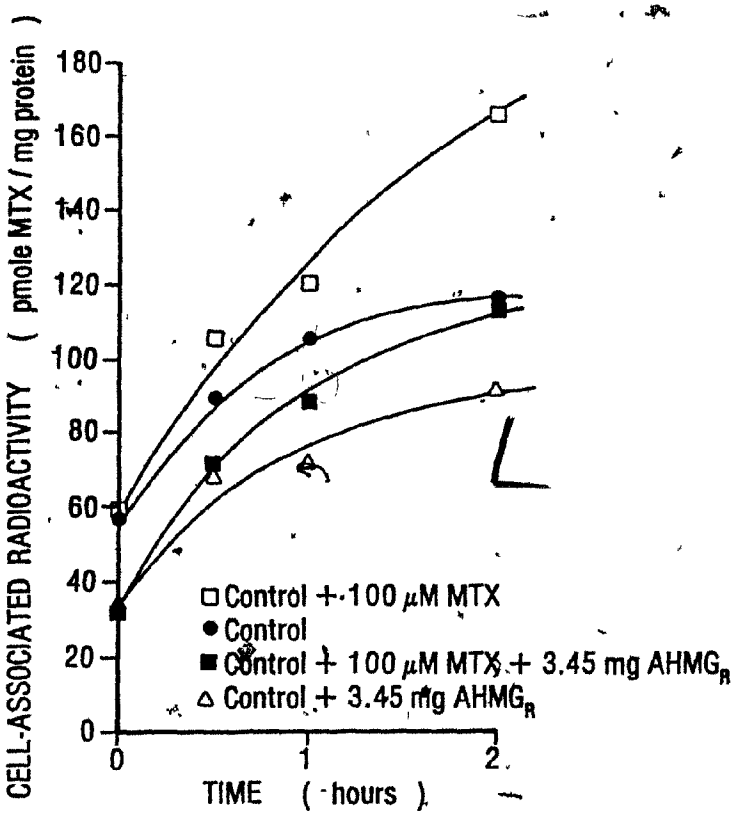
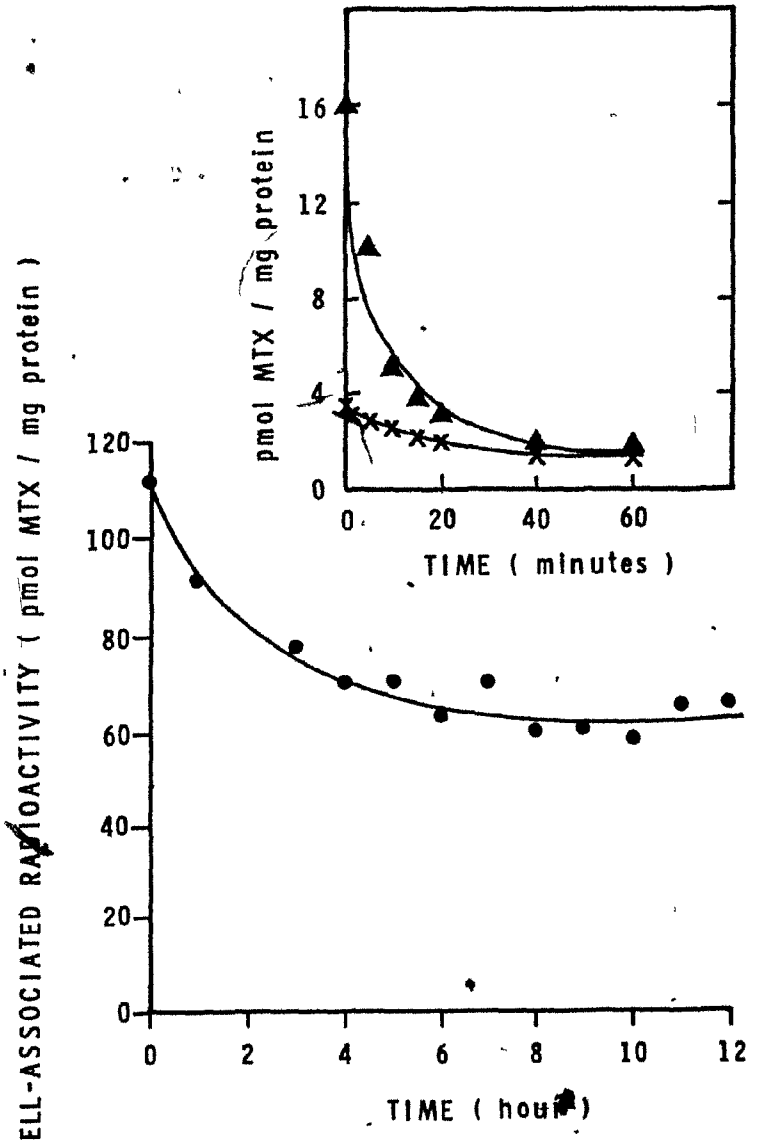

CELL-ASSOCIATED RADIOACTIVITY (pmole MTX / mg protein)
180
160
140
120
100
80
60
40
20
0
0
1
2
TIME (hours)
□ Control + 100 μM MTX
● Control
■ Control + 100 μM MTX + 3.45 mg AHMG$_R$
△ Control + 3.45 mg AHMG$_R$

Figure 15: EFFLUX FROM M21 CELLS PRELOADED WITH MTX-AHMG$_R$.

Cells (3 x 10^6 per culture dish, grown for 72 hr and washed) were incubated for 7.5 hr at 37°C with [^{3}H]MTX-AHMG$_R$ to give a concentration of 10 μM MTX in a total volume of 3 ml of RPMI without serum. The loaded cells were washed and incubated in medium without conjugate. At the times indicated, cells were washed again once, were dissolved in SDS-containing buffer and cell-associated radioactivity was measured as described in Materials and Methods. Points represent the mean of 3 determinations. Inset: Efflux from cells loaded by incubation at 37°C for 1 hr in the presence of [^{3}H]MTX at 1 (x) or 10 (▲) μM.


pmol MTX / mg protein
16
12
8
4
0
0
20
40
60
TIME (minutes)
CELL-ASSOCIATED RADIOACTIVITY (pmol MTX / mg protein)
120
100
80
60
40
20
0
0
2
4
6
8
10
12
TIME (hour)

administered radioactivity per g wet weight). Kidney, spleen, lung and tumor took up lesser amounts (2.0%, 1.6%, 0.6% and 0.5%, respectively) and the lowest uptake was in brain (0.06%). Unlike the EL4 cell, the M21 cell was unable to retain a substantial portion of the MTX taken up; at 24 hr, MTX had declined from its peak value of 0.5% at 30 min to 0.06% of the administered radioactivity per g wet weight. The level of MTX in the serum declined from its peak value of 1.4% per ml at 30 min to 0.02% per ml at 24 hr. At all time intervals > 90% of radioactivity was associated with the supernatant after precipitating with 10% TCA the sera of mice administered free MTX. In all tissues, the major part of the accumulated MTX was cleared by 3 hr and 1% or less remained at 24 hr. Like the EL4 lymphoma bearing mice, the peak level of uptake of MTX in brain was much lower than in the M21 tumor cells, but in contrast to the EL4 cells, its rate of clearance (judged by comparing radioactivities expressed as percentages of the peak activities) was much slower than the M21 tumor cells, as well as all the other normal tissues studied (declining from 0.06% of the administered dose per g wet weight to 0.03% by 3 hr and remaining at that level at 24 hr).

(ii) TISSUE DISTRIBUTION OF MTX-AHMG$_{R+T}$ AND MTX-NRG

The uptake of MTX-AHMG$_{R+T}$ and MTX-NRG conjugates by the M21 cells was similar throughout the observation period of 24 hours. The amount of radioactivity in tumor cells from animals given either MTX-AHMG$_{R+T}$ or MTX-NRG conjugate increased progressively until 3 hr after administration, those cells contained about 1.1% of the

administered dose per g wet weight. This level was sustained for the rest of the 24 hr observation period. When the peak level of uptake of MTX, MTX-AHMG$_{R+T}$ and MTX-NRG were compared, the level of MTX attained when it was administered as the conjugates was 2.5 times that of MTX administered free. Serum levels of both conjugates declined rapidly, however, throughout the 24 hr period of observations the serum levels of mice that received one of the conjugates were higher than those of mice that received free MTX. At all time intervals more than 90% of radioactivity was associated with the precipitate obtained after the sera of mice administered with either conjugate were precipitated with 10% TCA.

The patterns of uptake of the two conjugates were also similar in all the other tissues studied, except that the MTX-NRG conjugate tended to decline faster from the tissues than the MTX-AHMG$_{R+T}$ conjugate. In all tissues, except the intestine, conjugated MTX persisted throughout the 24 hr observation period, unlike free MTX which was cleared very quickly (except perhaps in the brain). Also, except in the intestine, the extent of uptake of the conjugates in all the tissues exceeded that of free MTX and these higher levels also persisted longer.

When the results of uptake of these three agents were expressed as pmole of MTX per mg of M21 cell protein, all the uptake levels of the conjugates exceeded the intracellular DHFR whereas the uptake of MTX was equal to the intracellular DHFR level at 24 hr (Figure 17).

G. CATABOLISM OF MTX-AHMG$_R$ BY M21 CELLS.

When cells are preloaded with conjugate for 7.5 hr and then incubated in conjugate-free medium for 9.5 hr, gel chromatography revealed low-molecular-weight MTX-containing fragments both in the cell homogenate and efflux medium (Figure 18). Fragments from both sources were capable of inhibiting DHFR. Fifty percent inhibitory concentrations for the intact conjugate, cell homogenate fraction, efflux medium fraction and free MTX were 6.3 pmol, 6.6 pmol, 5.7 pmol and 3.0 pmol, per 3 ml respectively (Table 2).

The low-molecular-weight fraction from the efflux medium was further resolved into 2 peaks by DEAE-cellulose chromatography (Figure 19). Both peaks migrated on thin layer chromatography with an R_f of 0.2 whereas MTX exhibited an R_f of 0.63 and the conjugate remained at the origin. The solvent system was 0.1 M glycine containing 2% EDTA, pH 9.0.

To investigate the possible role of lysosomal enzymes in catabolism of drug-antibody conjugates, M21 cells were incubated with MTX-AHMG$_R$ with and without 100 μM chloroquine or 100 μM leupeptin. At 7.5 hr, the cells incubated in the presence of chloroquine had taken up 148 pmol MTX/mg protein and those incubated in its absence had taken up 154 pmol MTX/mg protein. At 7.5 hr and in the presence of 100 μM leupeptin, the cells took up 152 pmol MTX/mg protein but in its absence, they took up 177 pmol MTX/mg protein. When the cells in medium containing chloroquine were allowed to efflux for 9 hr, 127 pmol MTX/mg protein (86% of the initial value) remained

Figure 16: UPTAKE AND CLEARANCE OF FREE MTX, MTX-AHMG$_{R+T}$ AND MTX-NRG IN SERUM AND TISSUES OF M21 MELANOMA-BEARING NUDE MICE

Mice were inoculated s.c. with 2 x 10^6 M21 cells and 21 days later, were injected i.v. with 5 mg/kg dose of either free [^{3}H]MTX (●), [^{3}H]MTX-AHMG$_{R+T}$ (▲) or [^{3}H]MTX-NRG (△), in a total volume of 0.4 ml of PBS. At the times indicated, tissue samples were obtained and were counted for ^{3}H radioactivity as described in Materials and Methods. Tissue-associated radioactivity is expressed as the percentage of the administered dose per g wet weight of tissue (%/g) or for serum per ml (%/ml). Each value is the mean of determinations on 3 samples from different animals.

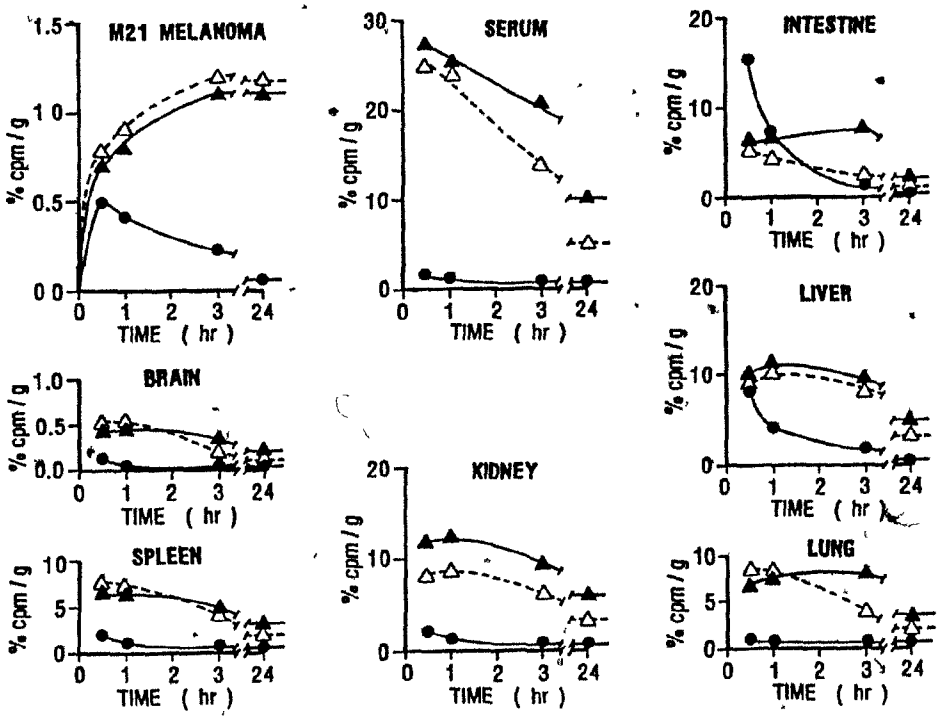

M21 MELANOMA
SERUM
INTESTINE
BRAIN
LIVER
SPLEEN
KIDNEY
LUNG
% cpm / g
TIME (hr)

Figure 17: UPTAKE OF FREE MTX (●), MTX-AHMG$_{R+T}$ (▲) AND MTX-NRG (△) BY M21 MELANOMA-BEARING NUDE MICE

The experimental conditions were as specified in Figure 16 but the results of determination of cell-associated radioactivity are expressed as pmol of MTX per mg protein. The dotted line shows the DHFR level in these cells, estimated from the data for efflux shown in Figure 15 inset.

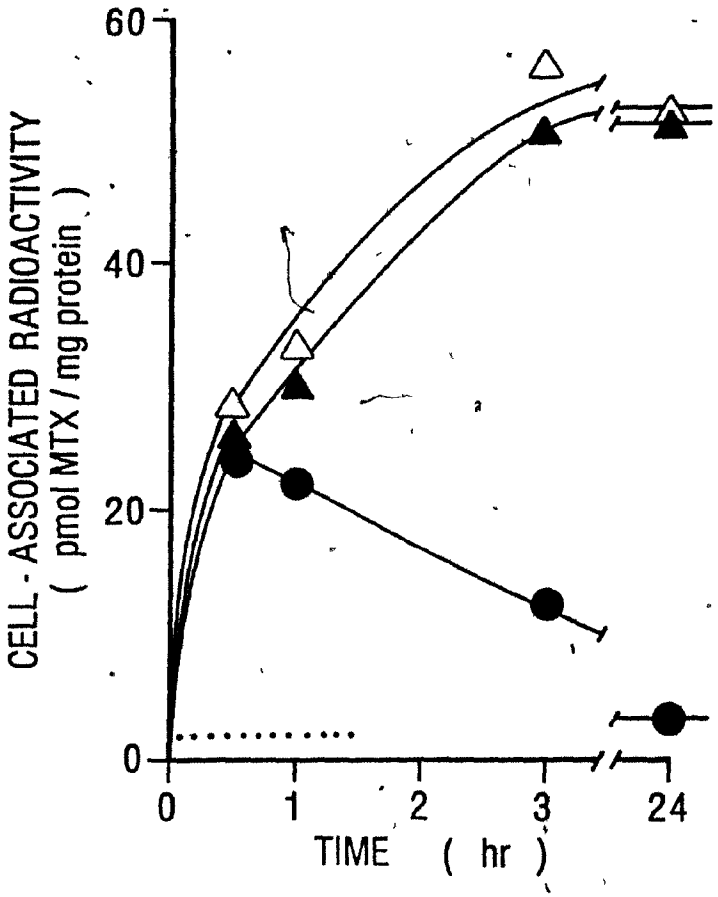

60
40
20
0
0
1
2
3
24
CELL - ASSOCIATED RADIOACTIVITY
(pmol MTX / mg protein)
TIME (hr)

Figure 18: GEL CHROMATOGRAPHY OF THE EFFLUX MEDIUM AND CELL HOMOGENATE AFTER LOADING M21 CELLS WITH [^{3}H]MTX-AHMG$_R$

The experimental conditions for loading and efflux were as specified in Figure 15. After 9.5 hr of efflux incubation in conjugate-free medium, cells were separated from medium by centrifugation and were homogenized. The homogenate was centrifuged and 1 ml of the supernatant was passed through a Biogel P100 column (1 x 20 cm) equilibrated with PBS; the medium used for efflux was lyophilized and was taken up in PBS, then a 1 ml sample was passed through the same column. Fractions of 1 ml were collected and were analyzed for radioactivity as described in Materials and Methods.

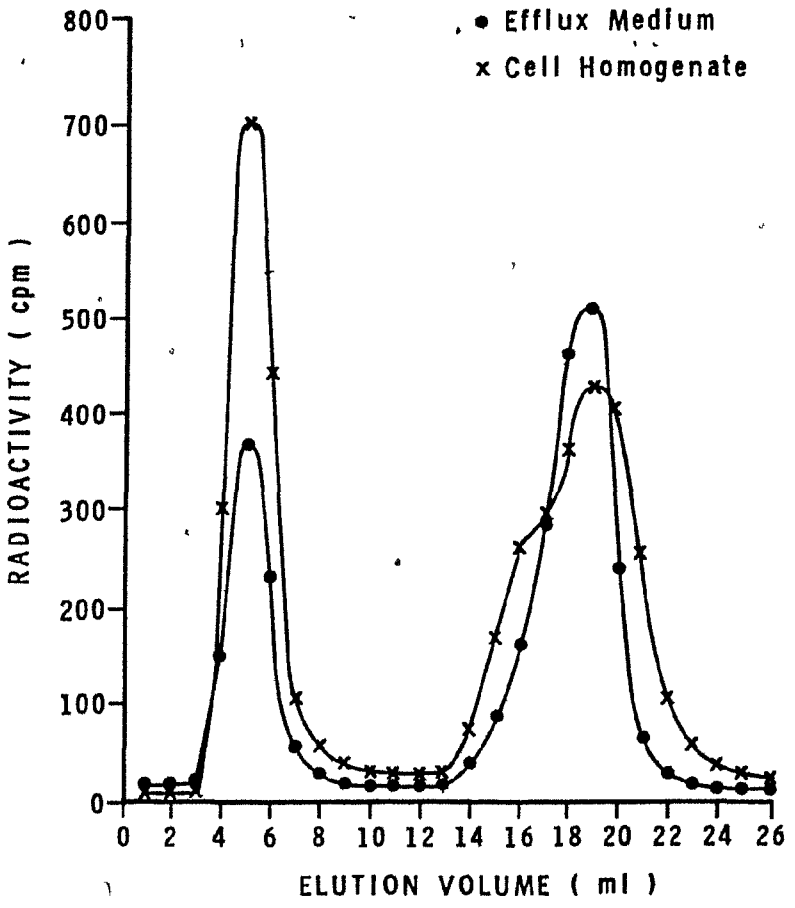

● Efflux Medium
x Cell Homogenate
RADIOACTIVITY (cpm)
800
700
600
500
400
300
200
100
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26
ELUTION VOLUME (ml)

Figure 19: DEAE-CELLULOSE CHROMATOGRAPHY OF LOW-MOLECULAR WEIGHT FRACTION FROM EFFLUX MEDIUM

The low-molecular weight fraction obtained from the efflux medium of M21 cells previously incubated with MTX-AHMG$_R$ was adsorbed on a column (1.2 x 30 cm) of DEAE-cellulose equilibrated with 5 mM phosphate buffer pH 7.0 and eluted with a linear gradient of 1 litre of 5 mM phosphate buffer in the mixing chamber and 1 litre of 0.5M NaCl in the same buffer in the reservoir. Fractions of 19 ml were collected. Arrow indicates the position that free MTX elutes.

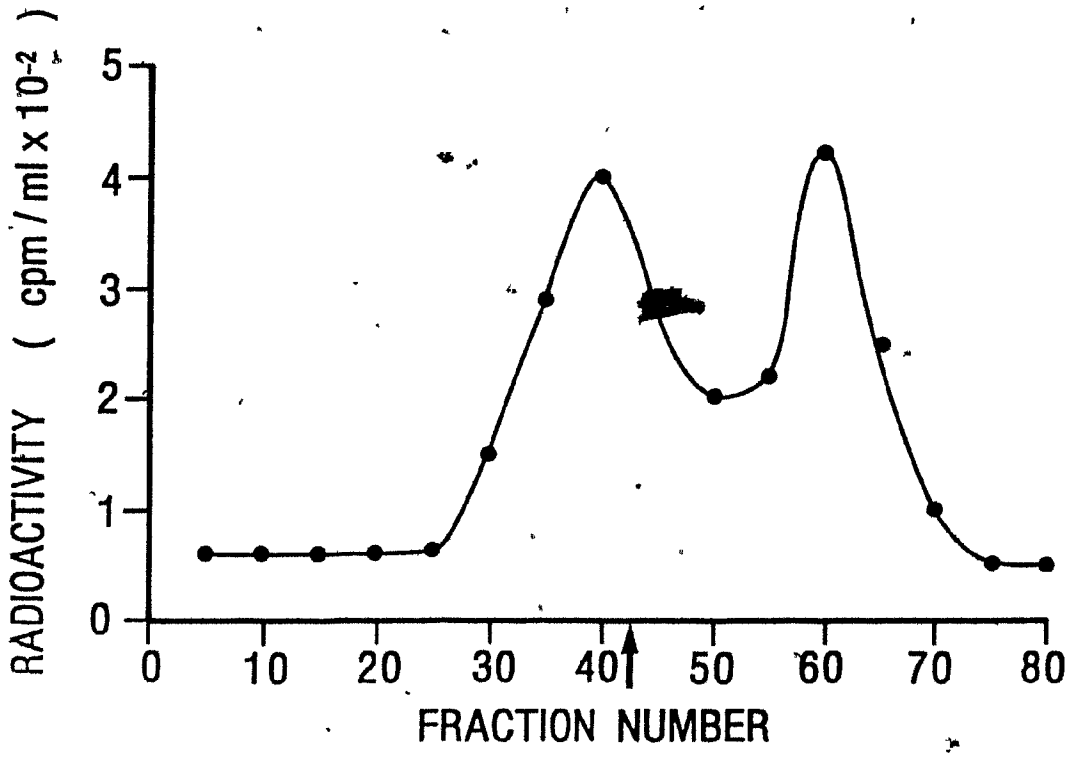

RADIOACTIVITY (cpm / ml x 10⁻²)
0
1
2
3
4
5
0
10
20
30
40
50
60
70
80
FRACTION NUMBER

TABLE 2: DHFR INHIBITION BY LOW MOLECULAR WEIGHT FRAGMENTS IN CELL EXTRACT AND EFFLUX MEDIUM

The assay method of Peterson et al., was used to determine the inhibition of DHFR by the low-molecular weight radioactivity-containing fractions from the cell extract and efflux medium. Reaction mixtures contained 0.5M sodium acetate buffer (pH 6.0), 0.6M KCl, 50 µM NADPH, 33 mM DHF and 0-15 pmol of MTX or corresponding amounts of test fractions in a total volume of 3 ml.

Table 2

DHFR INHIBITION BY LOW MOLECULAR WEIGHT FRAGMENTS IN CELL EXTRACT AND EFFLUX MEDIUM

Agent	Concentration for 50% inhibition of DHFR (pmole/3ml)
MTX	3.0
MTX-AHMG$_R$	6.3
Cell extract fraction	6.6
Efflux medium fraction	5.7

cell-associated, whereas, 92 pmol MTX/mg protein (60% of the initial value) remained in the cells that were preloaded and allowed to efflux in medium without chloroquine. With cells in medium containing leupeptin, 83.5 pmol MTX/mg protein (55% of the initial value) remained cell-associated after efflux for 9 hr and 54 pmol MTX/mg protein (31% of the initial value) remained in the cells that were preloaded and allowed to efflux in medium without leupeptin (Table 3).

H. DETERMINATION AND CHARACTERIZATION OF THE HYDROLYTIC ACTIVITY OF LIVER HOMOGENATES ON THE MTX-NRG CONJUGATE

When liver homogenates from adult C57BL/6J mice were tested for their ability to catabolize the MTX-NRG conjugate, a low molecular weight radioactive peak eluting slightly ahead of that for free [^{3}H]MTX was observed when the incubation mixture was passed through Biogel P100 (Figure 20). The catabolic activity was increased at low pH (Table 5) and by dialysing the homogenate overnight in buffer containing DTT (Table 4) but it was inhibited by leupeptin, pepstatin, antipain, N α-p-tosyl-L-lysine chloromethyl ketone and iodoacetate (Table 4), (Figure 21). Calcium chloride and EDTA had no effect on the activity (Table 4).

An attempt was made to further purify this low-molecular-weight fraction by adsorbing it on DEAE-cellulose and eluting with a linear gradient of 0-0.5 M NaCl in phosphate buffer. This resulted in the emergence of a peak that eluted at the same ionic strength as free MTX (Figure 22). When subjected to thin layer chromatography, using 0.1 M glycine containing 2% EDTA pH 9.0 as the solvent system, this eluted

fraction migrated with an R_f of 0.4 while MTX had an R_f of 0.63 and the conjugate remained at the origin.

I. IN VITRO INHIBITION OF GROWTH OF EL4 AND M21 CELLS

When EL4 cells were incubated with free MTX and its conjugates for 72 hr, the following concentrations for 50% inhibition of growth was obtained: free MTX, 1.54 nM, MTX-AELG, 220 nM, MTX-NRG, 550 nM. With M21 cells, the corresponding concentrations were: free MTX, 11.0 nM, MTX-$AHMG_R$, 220 nM, MTX-NRG, 660 nM (Table 6). Thus, MTX is a better inhibitor of tumor growth of both cell types in tissue culture than its conjugates.

TABLE 3: RESIDUAL RADIOACTIVITY ASSOCIATED WITH M21 CELLS INCUBATED WITH MTX-AHMG$_R$ IN THE PRESENCE OF CHLOROQUINE OR LEUPEPTIN

Cells were incubated with MTX-AHMG$_R$ and transferred to conjugate free medium as described in Figure 15 except that 100 μM chloroquine or leupeptin was present both in the preloading medium and the efflux medium. Control cells were incubated without chloroquine and leupeptin.

Table 3

RESIDUAL RADIOACTIVITY ASSOCIATED WITH M21 CELLS INCUBATED WITH MTX-AHMG$_R$ IN THE PRESENCE OF CHLOROQUINE OR LEUPEPTIN

Treatment of Cells	Initial level of Radioactivity* (pmol MTX/mg protein)	Residual Level of Radioactivity after efflux for 9 hr (pmol MTX/mg protein)	% of radioactivity remaining in the cells after efflux
Control	153.6	92.4	60
+ 100 μM Chloroquine	148.1	127.3	86
Control	177.4	54.4	31
+ 100 μM Leupeptin	152.1	83.5	55

*Level of radioactivity in the cells prior to efflux.

Figure 20: BREAKDOWN OF MTX-NRG CONJUGATE AFTER INCUBATION WITH LIVER HOMOGENATES

Freshly removed liver (2.2 g wet weight) from an adult female C57BL/6J mouse was homogenized in 10 ml of 0.1 M sodium acetate buffer pH 4.6 for determination of the hydrolytic activity of the liver homogenates. A typical reaction mixture consisted of; 0.5 ml MTX-NRG conjugate, 0.5 ml liver homogenate, and 1.2 ml of 0.1 M acetate buffer pH 4.6 in a total volume of 2.2 ml. The reaction mixture was incubated at 37°C for 12 to 28 hr. At the end of the incubation, the mixture was centrifuged and 1 ml of the supernatant was passed through a Biogel P100 column (1 x 20 cm) and eluted with 0.1M acetate buffer pH 4.6. Fractions (1 ml) were collected and counted for radioactivity.

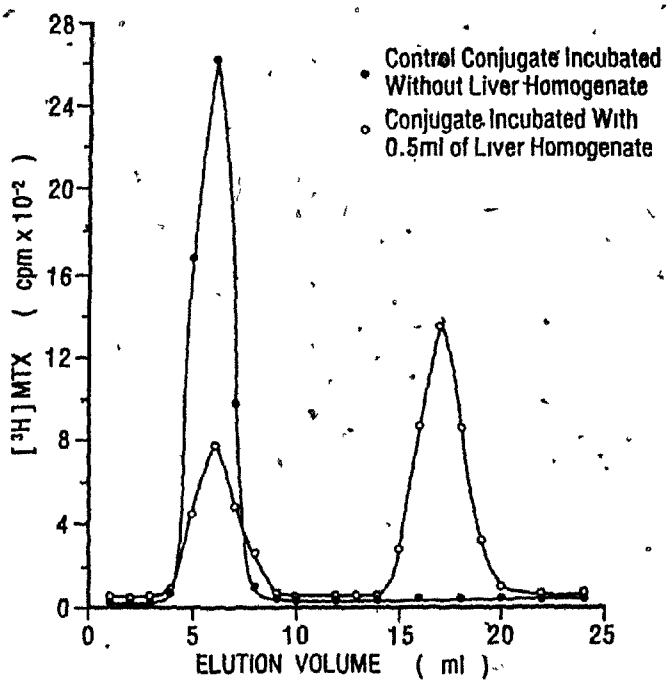

Control Conjugate Incubated
Without Liver Homogenate
Conjugate Incubated With
0.5ml of Liver Homogenate
[3H]MTX (cpm x 10-2)
28
24
20
16
12
8
4
0
0
5
10
15
20
25
ELUTION VOLUME (ml)

Figure 21: EFFECTS OF ANTIPAIN AND N-α-P-TOSYL-L-LYSINE CHLOROMETHYL KETONE ON HYDROLYSIS OF CONJUGATE BY MOUSE LIVER HOMOGENATES

The reaction conditions and assay of radioactivity were as in Figure 20 except that reaction mixtures contained 8.6×10^{-4}M N-α-p-tosyl-L-lysine chloromethyl ketone or 9.4×10^{-5}M antipain. Control, indicates conjugate incubated with liver homogenate as in Figure 20.

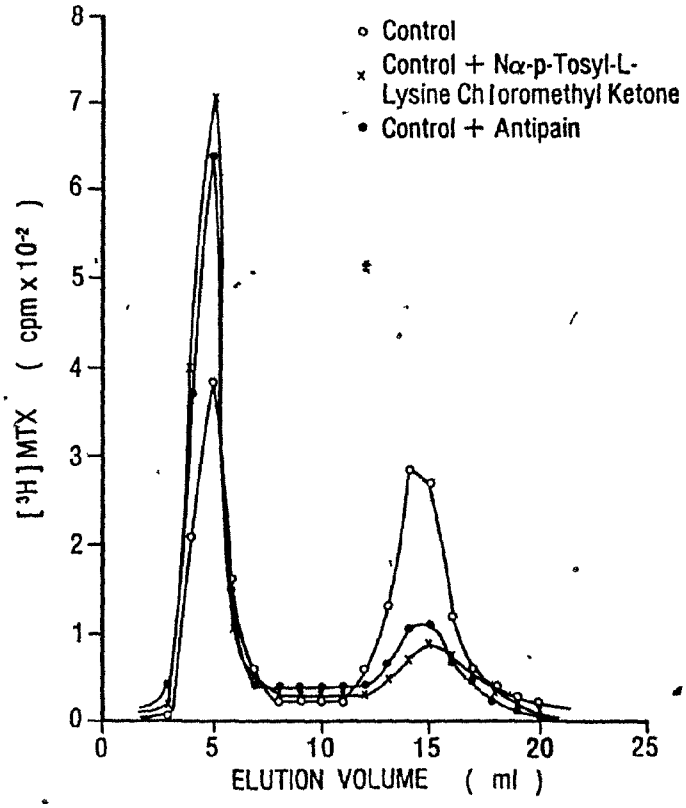

Control
Control + Nα-p-Tosyl-L-Lysine Chloromethyl Ketone
Control + Antipain
[³H]MTX (cpm x 10⁻²)
ELUTION VOLUME (ml)
0
1
2
3
4
5
6
7
8
0
5
10
15
20
25

TABLE 4: EFFECT OF SEVERAL INHIBITORS AND AN ACTIVATOR OF LYSOSOMAL ENZYMES ON THE BREAKDOWN OF MTX-NRG CONJUGATE BY LIVER HOMOGENATE

The reaction conditions and assay of radioactivity were as in Figure 20 except that reaction mixtures contained one of the following: leupeptin, pepstatin, antipain, Nα-p-tosyl-L-lysine chloromethyl ketone, calcium chloride, iodoacetate, EDTA, EDTA + DTT and DTT.

Table 4

EFFECT OF SEVERAL INHIBITORS AND AN ACTIVATOR OF LYSOSOMAL ENZYMES ON THE BREAKDOWN OF MTX-NRG CONJUGATE BY LIVER HOMOGENATE

Inhibitors	Concentration	% of Original Radioactivity in low MW Peak	% Inhibition
CONTROL	-	49	-
LEUPEPTIN	9.2×10^{-5} M	23	53
PEPSTATIN	9.2×10^{-5} M	34	31
ANTIPAIN	9.4×10^{-5} M	23	53
Nα-P-TOSYL-L-LYSINE CHLOROMETHYL KETONE	8.6×10^{-4} M	20	59
CALCIUM CHLORIDE	0.64×10^{-3} M	49	-
IODOACETATE	0.64×10^{-3} M	33	33
EDTA	0.64×10^{-3} M	49	-
EDTA + DITHIOTREITOL	0.64×10^{-3} M	62	27*
DITHIOTREITOL	0.64×10^{-3} M	59	20*

*ACTIVATION

TABLE 5: INFLUENCE OF pH ON THE BREAKDOWN OF MTX-NRG CONJUGATE BY LIVER HOMOGENATE

The reaction conditions and assay of radioactivity were as in Fig. 20 except that the reaction mixtures were incubated at 3 different pH values.

Table 5.

INFLUENCE OF pH ON THE BREAKDOWN OF MTX-NRG CONJUGATE BY LIVER HOMOGENATE

pH	% OF RADIOACTIVITY IN LOW M.W. PEAK
5.6	51.2
4.6	73.5
4.0	74.6

Figure 22: DEAE-CELLULOSE CHROMATOGRAPHY OF LOW MOLECULAR WEIGHT FRACTION FROM MTX-NRG INCUBATED WITH LIVER HOMOGENATES

The low-molecular weight fraction obtained from MTX-NRG incubated with liver homogenates was adsorbed on a column (1.2 x 30 cm) of DEAE-cellulose equilibrated with 5 mM phosphate buffer pH 7.0 and eluted with a linear gradient of 1 litre of 5 mM phosphate buffer in the mixing chamber and 1 litre of 0.5 M NaCl in the same buffer in the reservoir. Fractions of 19 ml were collected. Arrow indicates the position that free MTX elutes.

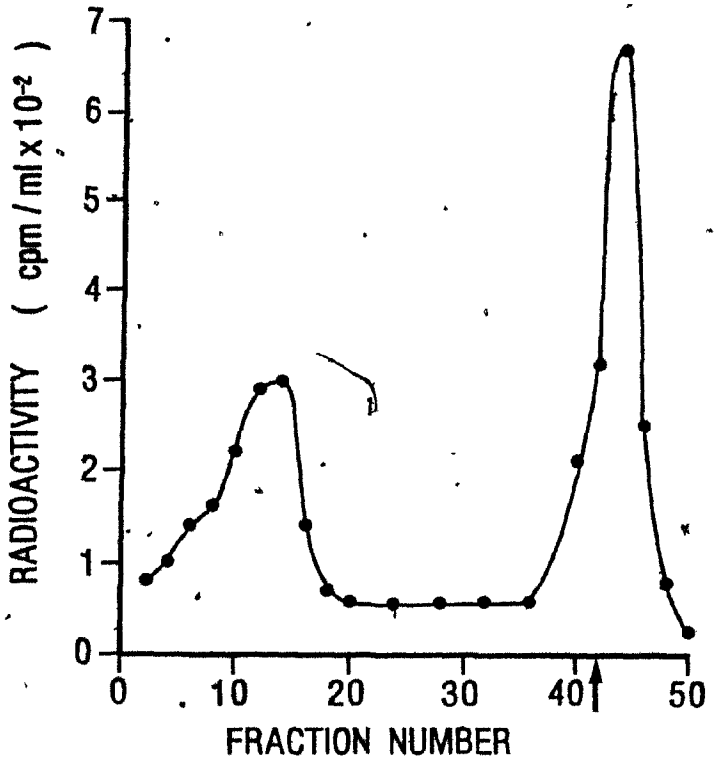

RADIOACTIVITY (cpm / ml x 10⁻²)
0
1
2
3
4
5
6
7
0
10
20
30
40
50
FRACTION NUMBER

TABLE 6: IN VITRO INHIBITION OF GROWTH OF EL4 AND M21 CELLS BY MTX; MTX-AELG, MTX-AHMG$_R$, MTX-NRG

EL4 cells obtained from the ascites fluid of C57BL/6J mice were kept in culture for 48 hr in RPMI 1640 medium supplemented with 10% fetal calf serum. At 48 hr, the cells were plated at a concentration of 0.5 x 10^5 cells per ml in 35 x 10 mm tissue culture dishes. M21 cells from in vitro passage were similarly plated in 35 x 10mm tissue culture dishes at a concentration of 0.5 x 10^5 cells per ml in RPMI 1640 medium supplemented with 10% fetal calf serum. MTX in the free or conjugated forms at various concentrations was added to the EL4 and M21 cells 24 hr after plating and incubated for a further 72 hr, after which they were harvested and counted in a Coulter counter to determine the inhibition of growth.

Table 6

IN VITRO INHBITION OF GROWTH OF EL4 and M21 CELLS BY MTX, MTX-AELG, MTX-AHMG$_R$, MTX-NRG

Agent	Concentration for 50% inhibition of Growth (nM)	
	EL4 Cell	M21 Cell
MTX	1.54	11.0
MTX-AELG	220	-
MTX-AHMG$_R$	-	220
MTX-NRG	550	660

DISCUSSION

The basis of the superior tumor inhibitory effect of drug-antitumor antibody conjugates has been attributed to ether, (a) greater delivery of drug to the tumor cell, (b) longer plasma half life or (c) synergism between the drug and antibody (Ghose and Blair, 1978), however, the role of each of these factors in the mechanism of action of drug-antibody conjugates has not been fully elaborated. Therefore, to explain the mechanism of action of their superior antitumor effect, I have investigated the role of drug uptake and serum clearance in detail by comparing the uptake of free and conjugated MTX by tumor cells in vitro and in vivo as well as the pharmacokinetics of free and conjugated MTX in tumor-bearing animals. In addition, I have also studied the catabolism of MTX-IgG conjugates by tumor cells and liver homogenates and the capacity of the catabolic fragments to inhibit DHFR, the target enzyme of MTX. The tumors used for these investigations were the murine EL4 lymphoma ascites tumor and the human melanoma M21 solid tumor. The choice of the EL4 lymphoma was influenced by the observation of Kulkarni et al., (1981) that MTX-antiEL4 lymphoma antibody inhibits the growth of the tumor in EL4 lymphoma bearing mice. Since the EL4 lymphoma is not a human tumor, it was desirable to extend the study to the human melanoma M21 tumor which in contrast to the EL4 ascites tumor is also a solid tumor. Moreover the investigation with the EL4 lymphoma model was limited to the polyclonal antiEL4 antibody but the availability of the monoclonal antibody 225.28S against the melanoma cells afforded the

opportunity to compare (and contrast) the carrier abilities of monoclonal and polyclonal antimelanoma antibodies.

The polyclonal antiEL4 and antimelanoma M21 antibodies (obtained by immunization of rabbits with EL4 and M21 cells respectively) and the monoclonal antimelanoma antibody 225.28S were IgG molecules. The active ester intermediate method was used for coupling MTX to the IgG molecules because of its better efficiency of linkage and protein recovery compared to the more commonly used ECDI method, and the established antitumor effect of conjugates prepared by this method (Kulkarni et al., 1981).

All the in vitro uptake studies were done with an external MTX concentration, free or bound, of 10 μM because this concentration is sufficiently high so that the uptake of the conjugates by the cells can be clearly distinguished from background radioactivity. Moreover, since this concentration of free MTX did not saturate the MTX carrier transport sites on the cells, the contribution to the uptake of free MTX by other mechanisms such as low-affinity carrier sites or passive diffusion can be excluded (Goldman, 1982; Jolivet et al., 1983). Both the EL4 cells and M21 cells rapidly took up MTX from the medium, as has been observed for several human melanoma lines (Kufe et al., 1980; Gaukroger et al., 1983), and several mouse lymphomas and other tumors (Goldman et al., 1968; Sirotnak and Donsbach, 1976).

It has been shown that the factors that determine the uptake and intracellular steady-state level of free MTX include the availability of a transport system, the external drug concentration, the membrane

potential (Goldman et al., 1968), and the ionic milieu (Henderson and Zevely, 1980). The uptake and the level of accumulation of MTX-IgG conjugates are unlikely to be subject to the same control mechanisms as is free MTX; in other words they will have different transport systems, e.g. endocytosis of AELG, following capping (Guclu et al., 1975) or nonspecific uptake by pinocytosis, as is observed with various serum proteins, including IgG (Chu and Whiteley, 1980; Ghose et al., 1962). In fact, Chu and Whiteley (1980) have demonstrated that L1210 lymphoma cells take up MTX linked to bovine serum albumin or to non-tumor-specific immunoglobulins in this way, and Shen and Ryser (1984) have shown that the complex formed between a MTX-human serum albumin conjugate (MTX-HSA) and antiHSA serum is taken up by Fc-receptor bearing cells that are defective in MTX transport, whereas Fc-receptor negative cells did not take up the complex significantly.

Cells internalize macromolecules by fluid-phase or receptor-mediated endocytosis. Light microscopy was used initially to enumerate phase-lucent pinocytotic vesicles in mammalian cells (Lewis, 1931; Cohn, 1966). When electron microscopy first suggested that most cells contain submicroscopic pinocytotic vesicles (Palade 1956; Bennett 1956), pinocytosis was subsequently studied by introducing a variety of electron dense probes e.g., colloidal gold and thorium (Fedorko et al., 1968; Cohn et al., 1966; Gordon et al., 1965; Odor 1956), ferritin (Farquhar and Palade 1960), and peroxidases (Graham and Karnovsky, 1966) into the extracellular medium. The presence of

these probes within vesicles confirmed their origin at the cell's surface and demonstrated further that incoming vesicles fuse with independently labeled lysosomes both in vitro (Cohn et al., 1966; Gordon et al., 1965) and in vivo (Friend and Farquhar, 1967; Straus, 1964). However, one difficulty with some of these probes is that they can adsorb to the cell surface; labeling of an apparently intracellular pinocytotic vesicle in a thin section may really represent extracellular binding to a surface invagination, a fold or even an outgoing vesicle. Other problems with some of these probes which limit their use for quantitative work are (a) they are difficult to visualize (b) the total amount of cell-associated marker in a typical culture is 1% or less of the administered load and (c) the relative amount of interiorized versus surface-bound label may be small. Therefore, a variety of radiolabeled markers have been used. They include [^{3}H] sucrose (Wagner et al., 1971), [^{3}H]inulin (Bowers and Olszewski, 1972) and [^{125}I]polyvinylpyrrolidone (Williams et al., 1975). The interaction of horseradish peroxidase with cells in tissue culture illustrates all the features of fluid-phase uptake (Steinman and Cohn, 1972; Steinman et al., 1974). The enzyme does not bind to the cells' surface. Its uptake is proportional to the concentration of enzyme in the medium, proceeds continuously with time, and is blocked at 4°C. The endocytosed enzyme is visualized cytochemically in intracellular pinocytotic vesicles and lysosomes (Steinman, 1976).

Most ligands, however, are internalized by receptor-mediated endocytosis. The first step in this multiphase process involves

binding of the ligand to its receptor molecule functionally exposed at the cell surface. In most cases these receptors are distributed diffusely over the cell surface. This has been demonstrated by visualization of fluorescent labeled ligands such as α_2-macroglobulin, insulin, epidermal growth factor, transferrin and asialoglycoprotein (Maxfield et al., 1978; Sullivan et al., 1976; Geuze et al., 1982). Receptor-mediated endocytosis can be determined by administering an independent marker to measure and/or visualize fluid-phase uptake before and during administration of the ligand to determine if ligand binding affects this parameter. The internalization process has generally been analyzed by identification of the ligand at an intracellular site by various techniques. These techniques include electron microscopic identification of ligands labeled with colloidal gold or horseradish peroxidase (Willingham and Pastan, 1983), autoradiography (Amsterdam et al., 1979; Unanue et al., 1972), subcellular fractionation (Courtoy et al., 1982; Mellman, 1982) and by fluorescence microscopy with ligands conjugated to various fluorescent probes (Willingham and Pastan, 1983; Weitzman et al., 1979; Geisow et al., 1981; Sklar et al., 1982).

Several methods have been used to distinguish between the amount of adsorbed versus internalized ligand. Usually, the adsorbed ligand on the plasma membrane is selectively eluted by (a) adding some competing agent e.g., EGTA or EDTA; as shown by Tolleshaug (1981), Schwartz et al., (1982), Weigel and Oka (1984) that these reagents could elute off asialoglycoprotein from its receptor; (b) proteolytic

treatment, e.g., Mellman (1982) used subtilisin to degrade plasma membrane bound IgG, Youngdahl et al., (1979) eluted off transcobalamin II-cobalamin complex from the plasma membrane of human skin fibroblasts with trypsin, and Dickson et al., (1981) eluted off α_2-macroglobulin from the plasma membrane of fibroblasts with pepsin at pH 4; (c) low pH treatment e.g. human chorionic gonadotropin was dissociated from its receptor with Hanks' solution that had been adjusted with concentrated acetic acid to pH 3.9 (Ahmed et al., 1981); (d) reversible dissociation from surface binding sites e.g., Goldstein et al., (1976) dissociated bound LDL from their receptors with heparin; the interaction was believed to represent an ionic binding of positively charged amino groups on the protein component of LDL to negatively charged sulfate groups on the heparin.

Attempts to selectively elute off plasma membrane bound MTX-antibody conjugates from EL4 and M21 cells with 0.12 M citrate buffer pH 3.0 were unsuccessful. After incubating them for 30 min at 4°C, 90% of the cells became permeable to trypan blue (i.e. nonviable) and they still reacted positively on immunofluorescence assay indicating that the conjugates were still bound to the cells. Even though proteolytic enzymes have been used to degrade plasma membrane bound immunoglobulins, there are no reports on the integrity of the cell membrane after such treatment. Dissociating ligands from their receptors reversibly with heparin as in the case of receptor bound LDL or competing with EDTA as in the case of receptor bound asialoglycoprotein is attractive but equivalent applicable agents for

dissociating receptor bound IgG from intact cells are not available.

Using the fluid-phase uptake marker, horseradish peroxidase, Steinman and Cohn (1972) and Steinman et al., (1974) showed that it is not adsorbed to the cell surface and its uptake is not detectable at 4°C whereas, at 37°C it is easily detected cytochemically in intracellular pinocytotic vesicles and lysosomes indicating that uptake by fluid-phase pinocytosis at 4°C is insignificant. Ryser (1968) arrived at a similar conclusion for adsorptive pinocytosis after he observed that exposure of cells to [^{131}I]albumin at 2°C for 1 min or for 60 min gave identical results as a 1 min exposure at 37°C but as the time of exposure at 37°C increased the uptake of the albumin by the cells increased. Various investigators (Willingham and Pastan, 1983; Goldstein et al., 1976; Youngdahl et al., 1979) have demonstrated that binding of ligands occurs at 0°C without endocytosis being detectable at this temperature, whereas, at 37°C cells endocytose the bound ligands. Therefore, in my in vitro uptake studies, plasma membrane bound conjugates were distinguished from internalized ones by comparing cell-associated radioactivities of cells incubated at 0°C with those incubated at 37°C.

With EL4 cells, the uptake of MTX-AELG remained progressive while that of MTX-NRG and free MTX leveled off; at 6 hr, cells incubated with MTX-AELG endocytosed more MTX (40 pmol/mg protein) than did cells incubated either with MTX alone (30 pmol/mg protein) or with MTX-NRG (24 pmol/mg protein). These results show that the uptake kinetics of MTX conjugates are different from those of the free drug and therefore

it is unlikely that significant amounts of conjugates were bound or internalized by the transport system for MTX.

With M21 cells, net uptake of MTX when linked to any of the three specific antibodies (MAB, $AHMG_R$, $AHMG_{R+T}$) exceeded that obtained on incubation with equimolar amounts of free drug as early as within the first hour, whereas, the net uptake of MTX when linked to NRG barely exceeded that of free MTX during the period of observation. The inhibition of MTX-$AHMG_R$ uptake by $AHMG_R$ and the inability of free MTX to inhibit this uptake indicates that transport of the conjugate depends on the antibody moiety and confirms that it is unlikely that substantial amounts of conjugates were bound or internalized by the transport system for MTX. The efflux studies show that conjugated MTX was retained longer than free MTX during incubation in drug or conjugate-free medium. This indicates that the specific antibodies against TAAs used in this study are capable of achieving and maintaining higher intracellular concentrations of MTX than is possible with the free drug.

Cell-association kinetics of the three specific MTX-antiM21 antibody conjugates were bi-or multiphasic except for MTX-MAB which was linear at 37°C. Measurements of cell-associated radioactivity at 37°C will include both binding and uptake but those at 0°C should be of just binding since endocytosis does not occur at this temperature (Willingham and Pastan, 1983). At 0°C, the binding phase completed prior to the first measurement at 1 minute can be accounted for by virtually immediate occupancy of high affinity sites. The following

progressive increase in binding which leveled off around 4 hours may be explained in the case of MTX-MAB by the differential accessibility of the high-molecular-weight melanoma associated antigen (MAA) epitope. In the case of MTX-AHMG$_R$ and MTX-AHMG$_{R+T}$, additional factors would include diversity of antigens and polyclonality of antibodies in the preparations. Binding taking place relatively slowly at 0°C would be expected to proceed much faster at 37°C as shown by MTX-AHMG$_{R+T}$ which bound approximately 40% more within 1 minute at 37°C than at 0°C. This value of 31 pmol MTX/mg protein at 37°C was close to the plateau level of 36 pmol MTX/mg protein observed after 4 hour incubation at 0°C and may thus include a proportion of lower-affinity antibody that bound within the first minute at 37°C. In contrast, with MTX-AHMG$_R$, nearly the same amount (40 pmol/mg protein) bound within 1 minute at 0°C or 37°C but the initial binding at 37°C was followed by a very rapid rate of increase in cell-associated conjugate, reaching in the next few minutes a value equal to that observed after 6 hours at 0°C, i.e. saturating binding sites. The further increase at 37°C must therefore be due to endocytosis. It appears that at least a proportion of the high-affinity-antibody populations (about 58%) in AHMG$_R$ which showed binding to M21 cells within 1 minute regardless of temperature were lost during the absorption procedure for rendering AHMG$_R$ melanoma-specific. This is not surprising since approximately 90% of the IgG in AHMG$_R$ was usually removed during absorptions with normal human tissues to produce AHMG$_{R+T}$. MTX-MAB showed approximately the

same immediate binding at 0°C and 37°C, followed by rapid increases in cell associated conjugate at either temperature during the first hour. The initial rate of increase in cell-associated radioactivity at 0°C was somewhat slower than at 37°C-and declined to zero by 4 hours, but net uptake at 37°C continued linearly throughout the 6 hour observation period, in contrast to MTX-AHMG$_R$ and MTX-AHMG$_{R+T}$, which approached a plateau after 4 hours. This difference in behaviour at 37°C may reflect differences in turnover of receptors for the different antibodies.

The net amount of MTX that is cell-associated after incubation with a conjugate at 37°C will depend upon a number of factors that include (i) specific binding of the conjugate to TAA or other antigens and subsequent endocytosis (or shedding, if any), (ii) nonspecific binding to the cell surface and uptake by mechanisms independent of specific transport carriers, e.g., pinocytosis (Ghose et al., 1962; Chu and Whiteley, 1980), (iii) efflux of MTX-containing fragments produced by intracellular catabolism and (iv) catabolic release of MTX-containing fragments mediated by proteolytic enzymes on the surface of tumor cells. Thus, calculations for determining the net uptake of conjugates by subtracting the amount bound at 0°C or the amount bound initially at 37°C from the total cell-associated MTX at a given time are only approximations and will neither distinguish nonspecific from specific uptake nor take into account the amount effluxed. However, the negligible uptake of MTX-NRG by the M21 cells indicates that nonspecific pinocytosis was not a major pathway in the

uptake of specific conjugates. This contrasts with the behaviour of EL4 cells which took up larger amounts of MTX-NRG than M21 cells did.

It was interesting to observe that the net uptake at 37°C correlated with extent of high affinity binding (measured at 1 min at 0°C) and with immunofluorescence titre, the order being MTX-MAB>MTX-AHMG$_R$>MTX-AHMG$_{R+T}$. Further, when the high affinity binding at 1 min was subtracted from the 0°C plateau value at 6 hours yielding the number of available binding sites with lower affinity, again the same order was evident. Indeed, this was further supported by the initial (starting at 1 min) rates of binding at 0°C, their values expressed as pmol/hr/mg protein being 63 for MTX-MAB, 15 for MTX-AHMG$_R$ and 4.7 for MTX-AHMG$_{R+T}$. Although the content of specific antibody molecules differed for each conjugate, the concentration of specific antibody in each incubation greatly exceeded available cell surface binding sites because the incubations were performed with equal numbers of M21 cells and equal concentrations of conjugated MTX which contained excess antibody, about 0.35 mg IgG, which is 74-, 9- and 5- fold higher than the titre values of MTX-MAB, MTX-AHMG$_R$ and MTX-AHMG$_{R+T}$ respectively. Thus, the amount of conjugate bound at equilibrium would depend chiefly on the available binding sites and their total number on the cell surface should be indicated by the plateau values of binding at 0°C. From this it can be concluded that net uptake at 37°C was directly correlated to the number of binding sites.

The properties of the binding sites, especially their lateral

mobility and facility for internalization, may also be important in the uptake of conjugated drugs. The ratio of conjugate taken up at 6 hours at 37°C to the plateau level of binding at 6 hours at 0°C may indicate the effectiveness of cell-surface binding sites for internalization of that conjugate. These ratios were 2.1, 1.2 and 1.4 for MTX-MAB, MTX-$AHMG_R$ and MTX-$AHMG_{R+T}$ respectively. The ratio of 2.1 for the MAB conjugate demonstrates that twice the amount needed for saturating binding sites at 0°C was endocytosed during 6 hours. Thus, the MAB appears to be a better carrier for MTX against M21 melanoma cells because a larger number of cellular binding sites are present that are intrinsically more effective in its internalization. $AHMG_R$ differed from $AHMG_{R+T}$ only in the lesser extent to which non-tumor-specific antibodies were absorbed out and thus had access to binding sites additional to the MAA which bound $AHMG_{R+T}$. Greater binding of MTX-$AHMG_R$ at 0°C was in fact observed, as well as coresponding greater net uptake at 37°C. MAB is distinct from the major antibody populations in $AHMG_R$ and $AHMG_{R+T}$ since it continues to react with melanoma cells coated with conventional antisera (Ghose et al., 1982). This indicates that the binding sites for MAB were more efficient in internalizing the ligand than the binding sites for $AHMG_R$ and $AHMG_{R+T}$.

These in vitro uptake studies have shown that both EL4 and M21 cells take up more MTX when it is conjugated to the respective antiTAA antibodies than as the NRG conjugate or as the free drug after their exposure to these agents. Thus, this increased uptake of the

MTX-antitumor antibody conjugates could partly account for their superior antitumor effectiveness. However, since the in vitro uptake of these agents by the tumor cells may not reflect the in vivo uptake pattern, these studies were extended to tumor-bearing animals.

On administration of a 5 mg/kg dose of free or conjugated MTX i.p. to EL4 lymphoma-bearing mice, the EL4 cells took up the greatest amount of MTX when it was injected in the form of its AELG conjugate. This finding confirms the in vitro investigations, in which EL4 cells were incubated with free MTX and MTX conjugates at 0°C and 37°C and showed that uptake was greatest for the AELG-linked drug. Also in keeping with the in vitro results, administration of MTX as the NRG conjugate to tumor-bearing animals led to less uptake of drug by tumor cells than its administration as the AELG conjugate but more than as the free drug.

Following the i.p. injection of MTX and its conjugates into the ascites tumor-bearing mice, absorption from ascites fluid into blood and other tissues was rapid, peak levels being reached by the free drug within 1 hr and by either conjugate within approximately 3 hr. Conjugates were cleared more slowly from ascites fluid than was free MTX: both exhibited slightly higher levels at 1 hr than at 30 min, whereas the free drug showed progressive clearance from the time of the first measurement. Since samples of ascites fluid were collected from the side of the abdomen opposite to the site of administration, diffusion of conjugates in this viscous fluid may have been slower than that of free MTX. After 1hr both conjugates started declining,

but the clearance of MTX-AELG was somewhat slower than that of MTX-NRG. This may have been due to binding of AELG to antigen either on the EL4 cell surface or free in the ascites fluid, which would also explain the slightly lower levels of MTX-AELG in serum compared to MTX-NRG. However, the higher level of MTX-AELG in EL4 cells was not due simply to its higher level in ascites fluid. The superior carrier ability of AELG compared to NRG could be seen, for example, from the data obtained at 3 hr showing that tumor cells took up 3 times as much MTX conjugated to AELG as MTX conjugated to NRG, even though the ratio in the ascites fluid was only 1.6.

In most tissues free MTX reached a peak by 1 hr, then declined steadily to low levels. This decline was most pronounced in liver and kidney, where the initial peak had been almost 20% of the administered dose per g dry weight. In tumor cells the initial peak was 4% of the administered dose per g dry weight, and the rate of decline was slower than in other tissues: at 1, 3 and 24 hr, tumor cells contained approximately 1.2, 1.0 and 0.6 nmol MTX per g wet weight, respectively; the corresponding figures for ascites fluid were 21, 2.5 and 0.04 nmol per ml and those for serum were 5.3, 1.1 and 0.1 nmol per ml. Others have also reported that MTX-sensitive mouse tumors clear MTX activity more slowly than other tissues (Anderson et al., 1970; Bischoff et al., 1971; Dedrick et al., 1973; Henderson et al., 1965a).

Uptake of MTX linked to NRG by the EL4 cells can be attributed to these cells' high pinocytotic activity (Chu and Whiteley, 1980; Ghose

et al., 1962), and pinocytosis could also play a role in uptake of the AELG conjugate. However, an additional, more important uptake mechanism for MTX-AELG is likely to be capping and endocytosis at 37°C subsequent to binding of the AELG conjugate to specific receptors on the cell surface, as has been shown by in vitro experiments at 0°C and 37°C with AELG-carrying chlorambucil (Guclu et al., 1975). The fact that the level of immunoglobulin-linked MTX in ascites fluid and serum was persistently higher than the level of free MTX could also have contributed to higher initial uptake and persistence of high levels of MTX in the tumor cells.

However, studies with free MTX have shown that cytotoxic action correlates with the excess of drug over the stoichiometric DHFR level and the duration of exposure (Goldman, 1975). Maintenance of such high levels is facilitated by formation of polyglutamates that are retained better by the cell than is MTX itself (Fry et al., 1983; Goldman, 1975). The efflux studies gave a value for the DHFR level in these tumor cells of approximately 0.3 nmol per g wet weight; therefore, administering 5 mg free MTX/kg could maintain cytotoxic amounts for at least 24 hr. The more effective tumor inhibitory action reported by Kulkarni et al., for both MTX-AELG and MTX-NRG at this dose level may be due to the higher levels of intracellular drug now demonstrated to persist during the observation period of 24 hr. Another contributing factor could be sustained release of more potent free MTX by intracellular catabolism of the conjugate. Incubation of MTX-NRG with an EL4 homogenate produced a low molecular weight

[^{3}H]MTX-containing fragment. Studies on this and other similar MTX-containing catabolic products derived from MTX-IgG conjugates have shown them to be not more effective inhibitors of DHFR in vitro than the parent conjugates which are themselves approximately half as inhibitory as free MTX. The loss of potency of conjugated MTX could have been more than compensated for by the large excess of drug persisting in EL4 cells 24 hr after administration as a conjugate (e.g. 135 pmol MTX/mg protein in mice given MTX-AELG compared to 17 pmol MTX/mg protein in mice given free MTX). Measurements beyond 24 hr would have shown how long the excess (relative to the level of intracellular DHFR) produced by conjugate administration could be maintained in comparison with the level produced by free MTX administration. Unfortunately the EL4 lymphoma model has not allowed a longer period of observation. An interval of seven days between i.p. inoculation of 10^7 EL4 cells and sacrifice was necessary for the development of the ascites tumor and, after 7 days, the ascites fluid contained substantial numbers of red cells that also take up MTX (Steele et al., 1982). Further, tumor inoculated mice started dying after the 8th day. However, the results indicate that the higher plateau levels of the drug administered as the conjugate are likely to be maintained well beyond 24 hr.

Therefore, the general pattern of inhibition of tumor growth observed by Kulkarni et al., (1981), i.e. MTX-AELG>MTX-NRG>MTX corresponds to the uptake of these agents in vivo by the EL4 cells. In addition, the longer persistence of the MTX-IgGs in ascites fluid

and serum ensures continued delivery of drug to the tumor site thereby enhancing their therapeutic effectiveness compared to the free drug which was rapidly cleared from the circulation including the ascites fluid. The longer persistence of conjugated MTX in the circulation and in vital normal tissues may explain the remarkable increase in the systemic toxicity of this drug when administered in the conjugated form. The i.p. LD_{10} dose decreased from 15 mg/kg for free MTX to 5 mg/kg for either the AELG or NRG conjugates. Thus, in this model, the increase in antitumor effect of the MTX-NRG conjugate in vivo can be explained in part on the basis of longer persistence of conjugated drug in the circulation (and ascites fluid) and its greater uptake and longer retention by the EL4 cells. The superiority of MTX-AELG over MTX-NRG can be attributed to greater binding and still higher uptake of MTX when linked to AELG.

A recent investigation by Ghose et al., (1984) which showed that MTX-$AHMG_{R+T}$ inhibited melanoma M21 tumor growth significantly whereas, MTX-NMG or free MTX did not suggests that the $AHMG_{R+T}$ conjugate could be taken up more by the M21 cells than the NMG conjugate or the free drug. This is especially interesting as the drugs were administered by the i.v. route and the tumor was xenografted subcutaneously. This contrasts with the in vivo uptake of free and conjugated MTX in EL4 lymphoma-bearing mice in which the drugs were injected directly into the peritoneal cavity where the tumor cells were suspended in the ascites fluid, thus, favouring uptake, since the drugs and tumor cells were in direct contact. Since

the $AHMG_{R+T}$ conjugate has been demonstrated to be taken up more than either MTX-NRG or the free drug by M21 cells in vitro, the uptake pattern of these agents in melanoma M21-bearing mice was investigated to determine if this same sequence of drug uptake by the cells would be repeated in vivo.

This study demonstrated that MTX-$AHMG_{R+T}$ and MTX-NRG conjugates, when administered by the i.v. route, could be taken up by subcutaneous xenografts of human melanoma M21 cells. Though there was no difference in the uptake by the melanoma cells of the MTX-$AHMG_{R+T}$ and MTX-NRG, the amount taken up (approximately 55 pmol MTX per mg protein in 3 hr which remained constant up to 24 hr when observation ceased) was far in excess of the DHFR content of the cell (approximately 2 pmol MTX per mg protein), whereas free MTX declined rapidly from its highest uptake of 24 pmol MTX per mg protein at 30 min to 3 pmol MTX per mg protein at 24 hr, just above the DHFR content of the cell.

Since tumors have impaired lymphatic drainage (Ghose et al., 1976; Hoffer and Gottschalk, 1976) the MTX-NRG that reaches the extracellular compartment of the tumor might be trapped, thus resulting in a high concentration of the conjugate at the tumor site. Moreover, the uptake of the $AHMG_{R+T}$ conjugate could also be limited by its low content of melanoma specific antibody; this imposes a limit on the actual amount of melanoma specific antibody that diffuses to the tumor site. Thus, this conjugate preparation may have failed to differentiate specific uptake of MTX-$AHMG_{R+T}$ from nonspecific uptake

of MTX-NRG because of these technical difficulties.

This result contrasts with the observations of Ghose et al., (1984) which showed that MTX-AHMG$_{R+T}$ inhibited tumor growth significantly whereas, MTX-NMG or free MTX did not. However, in the study of the in vitro uptake of MTX and its conjugates by M21 melanoma cells, the order of drug taken up after exposure to these agents was MTX-MAB>MTX-AHMG$_{R+T}$>MTX=MTX-NRG, i.e. roughly the same as the order of tumor inhibition in vivo by these agents. The fact that these cells took up less MTX as the NRG conjugate in vitro indicates that they have a low pinocytotic activity. Hence the difference in the therapeutic responsiveness between MTX-AHMG$_{R+T}$ and MTX-NRG could be attributed to the higher internalization of the AHMG$_{R+T}$ conjugate at the tumor site. For the in vivo uptake studies mice were allowed to develop solid tumors with an average volume of 874 mm^3. Therefore, the MAAs in the tumor against which the AHMG$_{R+T}$ is directed are likely to be less exposed to the MTX-AHMG$_{R+T}$ than when the cells are in the form of a monolayer as they appear in vitro. In other words, the uptake of the MTX-AHMG$_{R+T}$ by M21 cells is greater in vitro because the MAA epitopes are more accessible to the conjugate in vitro than in vivo.

It is not surprising then, that the MTX-AHMG$_{R+T}$ is more effective in vivo in inhibiting the growth of tumor than MTX-NRG. In this study, mice were treated on days 1,4,7,10 and 14 with a 5 mg/kg dose of MTX, free or linked, 24 hours after subcutaneous inoculation of 2 x 10^6 M21 melanoma cells. The MTX-AHMG$_{R+T}$ was more effective in inhibiting tumor growth in the early days after tumor inoculation.

Its inhibitory effect decreased as the volume of the tumor increased; it inhibited mean tumor volume by 65% on day 14, the last day of treatment and by 15% on day 50. Though the inhibitory effect of the MTX-MAB was greater than that of MTX-$AHMG_{R+T}$, its pattern of inhibition was similar; it inhibited tumor growth by 90% on day 14 and 55% on day 50. The MTX-NMG had no inhibitory effect on tumor growth. These inhibitory effects correspond to the in vitro uptake studies and suggest a direct relationship between availability of binding sites and inhibition of growth. It also implies that as the tumor volume increases, fewer antigen binding sites are accessible to the antibodies resulting in less uptake and consequently less tumor inhibition.

There was a large difference in the serum concentration of the free and conjugated drugs; while the highest concentration of the free MTX was 1.4% of the administered dose per ml in 30 min, that of the conjugates were 27.4% per ml and 25.2% per ml for the $AHMG_{R+T}$ and NRG respectively. In addition to this initial large difference in serum concentration, there was a continuing rapid decrease in the serum concentration of free MTX and by the 24th hr, all the free drug had been eliminated, whereas, the serum levels of the conjugates declined gradually. At 24 hr, the serum level of MTX-$AHMG_{R+T}$ was twice that of the MTX-NRG. Thus, the high serum concentration attained by the conjugates (i.e. $AHMG_{R+T}$ and NRG) and which was maintained over a long time interval could ensure that adequate amounts of drug are continuously delivered to the tumor.

Greater than 90% of radioactivity was always associated with the precipitate obtained when sera from conjugate treated mice were precipitated with 10% TCA; therefore, the decline in the serum level of the conjugates is probably due to complete or partial hydrolysis followed by rapid elimination. Since MTX is known to bind to serum proteins (Henderson et al., 1965b; Steele et al., 1979b), [^{3}H]MTX released by hydrolysis of the conjugate could bind to these proteins thereby resulting in the consistently high levels of radioactivity obtained with the TCA precipitates. However, this is not likely because the TCA precipitates of sera of mice treated with free MTX always gave radioactivity of less than 5%.

High uptake of free MTX was observed in the liver and intestine. While the free MTX in the liver declined from its peak uptake level in 30 min to a barely detectable level in 24 hr, the free MTX in the intestine declined from its highest uptake level in 30 min to its lowest level in 3 hr but remained unchanged at this level up to 24 hr. This is consistent with the proposition that the bile could act as a reservoir for MTX which is being secreted and circulated in the enterohepatic route thereby resulting in the toxicity usually observed in liver and intestine (Bischoff et al., 1971; Calvert et al., 1977). Substantial uptake of free MTX was observed in spleen, kidney and lung with the brain having the lowest level of uptake. Similar observations have been made by Anderson et al., (1970) and these also correspond with the results from the EL4-bearing mice. However, in all the tissues mentioned above, the free MTX declined very rapidly

from its peak values in 30 min to barely detectable levels in 24 hr; indeed, by the third hour of observation all the MTX had been eliminated. In contrast, all the tissues showed consistently high uptake of the conjugates with the liver and kidney having the highest uptake and the brain the least. There was a gradual decline in the tissue content of MTX-$AHMG_{R+T}$ and MTX-NRG but at 24 hr substantial levels were still retained. In all the tissues, including serum, the NRG conjugate was being eliminated faster than the $AHMG_{R+T}$ conjugate.

Melanoma cells have been shown to be sensitive to MTX in vitro but resistant to this drug in vivo (Ghose et al., 1984; Fisher et al., 1979; Karakousis and Carlson, 1979). This paradoxical response has been attributed partly to the increased in vivo formation of MTX's 7-OH metabolite, which is two orders of magnitude less cytotoxic toward melanoma cells and competes with MTX for uptake (Gaukroger et al., 1983; Fabre et al., 1984). This study demonstrates that an additional factor to explain the lack of response of melanoma cells to MTX in vivo could be high efflux rate of the free drug from the cells in vivo. In contrast to the sensitive EL4 cells where 45% of the peak radioactivity remained associated with the cells at 24 hr, only 11.8% of the peak radioactivity remained associated with the melanoma cells at 24 hr. Hence, whereas in the EL4 lymphoma cells, there was enough MTX within the cells to keep DHFR highly inhibited, at least up to 24 hr, the melanoma cells on the other hand were unable to retain enough MTX to keep their DHFR in the inhibited state up to 24 hr.

The in vivo uptake studies conducted with the murine EL4 lymphoma

ascites tumor and the human melanoma M21 solid tumor have shown that MTX-IgG conjugates are taken up better by these tumors than the free drug. They also demonstrated that the conjugated drugs are retained longer in the circulation than the free drug. Therefore, greater delivery of the conjugated drug to the tumors and its longer plasma half life appear to have a significant role in the therapeutic effectiveness of MTX-IgG conjugates. However, the fate of the conjugated drug after internalization is still unknown. After internalization, the conjugates could either inhibit DHFR directly, or be hydrolyzed to release active MTX containing catabolites which could then inhibit DHFR or both the intact conjugate and its catabolic products could equally inhibit the enzyme. An investigation by Chu and Whiteley (1980) has shown that MTX-BSA was not degraded on the cell membrane of L1210 lymphoma cells but was degraded intracellularly. Similar investigations by Shen and Ryser (1979) and Galivan et al., (1982) with MTX-poly-L-lysine conjugates have shown that the complex was degraded intracellularly by Chinese hamster ovary cells and hepatic cells in culture respectively and that the degradation is sensitive to leupeptin and lysosomotropic inhibitors, thus implicating lysosomes in the breakdown process.

In this study the catabolic ability of M21 cells, EL4 cell homogenates and liver homogenates on MTX-IgG were examined and its degradation products partially characterized. Liver homogenates were used as a source of lysosomal enzymes and as a control against which the effects of different inhibitors of lysosomal enzymes on the

breakdown of the MTX-IgG by the homogenates could be tested.

After M21 cells were loaded with [^{3}H]MTX-AHMG$_R$ and incubated in conjugate-free medium, MTX-containing low molecular-weight catabolites were detected intracellularly and in the efflux medium. This catabolic process was inhibited with chloroquine and leupeptin. Incubation of MTX-NRG with EL4 cell homogenates or with adult C57BL/6J liver homogenates also produced a MTX containing catabolite fraction with similar chromatographic behaviour on Biogel P100 as that produced by M21 melanoma cells. This catabolic process was inhibited by all the five known inhibitors of lysosomal enzymes tested and was increased at low pH. The low molecular-weight radioactive fragments obtained after the incubation of [^{3}H]MTX-NRG conjugate with mouse liver homogenates or from the extract of M21 cells and their efflux medium after incubation of M21 cells preloaded with [^{3}H]MTX-AHMG$_R$ were as effective in inhibiting DHFR in vitro as the parent conjugates which in turn were half as effective as MTX. This result is in agreement with the investigations of Rosowsky et al., (1984) who found that derivatives of MTX in which the α-carboxyl group is joined to the ε-amino group of L-lysine, L-lysyl-L-lysine or L-lysyl-L-lysyl-L-lysine were 3-fold less effective in inhibiting DHFR compared to MTX in a cell free assay system.

The low-molecular-weight fraction from the efflux medium of M21 cells preloaded with MTX-AHMG$_R$ was further resolved into 2 peaks by DEAE-cellulose chromatography. One of the peaks eluted at the same ionic strength as free MTX but the other eluted at a slightly higher

ionic strength. This indicates that the efflux medium contained two differently charged catabolic products. The fraction that eluted at the higher ionic strength is not likely to be a polyglutamate derivative because it is two fold less effective in inhibiting DHFR than free MTX; polyglutamates of MTX are as effective as free MTX in inhibiting DHFR (Jacobs et al., 1975; Clendeninn et al., 1983). The charge difference between the two fractions was not enough to differentiate them on TLC; they both migrated with an R_f of 0.2 while free MTX had an R_f of 0.63 and the original conjugate remained at the origin.

The low-molecular-weight fraction from MTX-NRG incubated with liver homogenates was also further purified using DEAE-cellulose chromatography. The fraction that eluted at approximately the same ionic strength as MTX was also two fold less effective in inhibiting DHFR than free MTX but migrated with an R_f of 0.4 on TLC. These charge differences in the catabolic products obtained from the incubation of MTX-NRG with liver homogenates and from the efflux medium of M21 cells preloaded with MTX-AHMG$_R$ might be related to differences in amino acids still attached to MTX.

This study has established that the MTX-IgG conjugate is hydrolyzed intracellularly to release MTX containing fragment that also inhibits DHFR. Hence, reasons for the increase in cytotoxic potential of MTX-IgG conjugates in vivo would include higher uptake of the conjugate followed by slow sustained catabolism so that there is prolonged maintenance of intracellular active derivatives (i.e. intact

conjugate and its catabolites) at a level higher than that of DHFR.

Since both EL4 and M21 cells take up more MTX as the antiTAA antibody conjugate than as the NRG conjugate or free MTX it would be expected that the antiTAA antibody conjugate would be more inhibitory to the growth of these cells in culture. However, when their toxicities to these cells grown in RPMI 1640 medium supplemented with 10% fetal calf serum were tested, free MTX was observed to be a more potent inhibitor of growth of EL4 and M21 cells than when it was linked to either AELG, $AHMG_{R+T}$ or NRG. Similar observations have been made by Chu et al., (1981), Chu and Whiteley (1980) and Arnold et al., (1983). However, the best cytotoxicity assay may be to expose the cells to both free and bound MTX for a sufficient time to allow uptake; then after washing off unbound drug, the cells are grown in soft agar. The advantage of this assay is that it is comparable to the in vivo situation where free MTX is subject to a rapid efflux in the absence of the drug in the environment. The higher cytotoxicity of free MTX to cells in vitro may be related to its conversion to MTX polyglutamates which are capable of inhibiting other intracellular enzymes, as shown recently by Allegra et al., (1984). In addition to inhibiting DHFR, MTX polyglutamates also inhibited thymidylate synthetase, aminoimidazolecarboxamide ribonucleotide transformylase and methylene tetrahydrofolate reductase; free MTX did not inhibit these other enzymes apart from

DMFR. It is not known, however, if MTX-IgG or fragments generated from it also inhibit these additional enzymes.

CONCLUSION

1. In the EL4 lymphoma system more MTX was taken up both in vitro and in vivo when MTX was conjugated to antitumor IgG than when tumor cells were exposed to drug alone or drug linked to NRG.
2. More MTX was taken up in vivo by EL4 cells when the drug was linked to NRG than when the tumor cells were exposed to the free drug. In vitro, both are taken up to the same extent at 6 hr.
3. The extent of drug uptake by tumor cells after exposure to the agents in vivo correlated with their tumor inhibiting effect in vivo.

4(i) In EL4 lymphoma-bearing mice, the clearance from serum and tissue of MTX-IgG conjugates was slower than that of free drug.

(ii) In EL4 lymphoma-bearing mice, the tumor cells retained higher amounts of drug for a longer period when administered as the antitumor IgG conjugate. In contrast, no significant difference in clearance between MTX-NRG and MTX-antitumor IgG conjugates was observed in normal tissues examined.

5. Homogenates of EL4 cells could break down MTX-NRG conjugate into small drug-containing fragments.
6. In the human melanoma system in vitro, more MTX was taken up when MTX was conjugated to antitumor IgG than when the tumor cells were exposed to drug alone or drug linked to NRG. In this model there was a positive correlation between the number of available binding sites for antibody and the amount of drug taken up. However, in unlike the EL4 system, there was no difference in the

uptake of MTX by M21 cells in vivo when the drug was conjugated to either antitumor IgG or NRG.

7. There was no difference in the uptake of MTX either as the free drug or as the NRG conjugate by M21 cells in vitro.

8. The extent of drug uptake by M21 cells after exposure to the agents in vitro correlated with their tumor inhibitory effect in vivo.

9(i) In M21 melanoma-bearing nude mice, the clearance from serum and tissue of MTX-IgG conjugates was slower than that of free drug.

(ii) In M21 melanoma-bearing nude mice, the tumor cells retained higher amounts of drug for a longer period when administered as the IgG conjugates. There was no significant difference in the clearance of the IgG conjugates from normal tissues except that the clearance of the NRG conjugate in serum was faster than that of the antitumor IgG conjugate.

10. Efflux from tumor cells of IgG-linked MTX was much slower than that of free MTX.

11(i) M21 cells could break down the conjugate into small drug-containing fragments that could be detected both intracellularly and in the efflux medium. This catabolic process was inhibited with chloroquine and leupeptin, i.e. inhibitors of lysosomal enzymes.

(ii) Incubation of MTX-NRG conjugate with adult C57BL/6J liver homogenates also produced a MTX containing fragment similar to that produced by M21 melanoma cells. This catabolic process was

inhibited by all the 5 known inhibitors of lysosomal enzymes tested and was optimal at low pH.

12. The low molecular-weight radioactive fragments obtained after the incubation of [^{3}H]MTX-NRG conjugate with mouse liver homogenates or from the extract of M21 cells and their efflux medium after incubation of M21 cells preloaded with [^{3}H]MTX-AHMG$_R$ were as effective in inhibiting DHFR as the parent conjugates.

Therefore, reasons for the increase in cytotoxic potential of MTX-IgG conjugates in vivo would include higher uptake of IgG-linked MTX followed by slow sustained catabolism so that there is prolonged maintenance of intracellular active derivatives at a level higher than that of DHFR.

REFERENCES

1. Ahmed CE, Sawyer, HR and Niswender GD, 1981, Endocrinology, 109, 1380.

2. Alexander P, Delorme EJ, Hamilton LDG and Hall JG, 1967, Nature, 213, 569.

3. Allegra CJ, Drake JC, Jolivet J and Chabner BA, 1984, Proc. Am. Assoc. Cancer Res., 25, 21 abstract.

4. Amenta JS, Baccino FM and Sargus MJ, 1977a, in: Intracellular Protein Catabolism, eds. Turk V and Marks N (Plenum Press, New York) p. 27.

5. Amenta JS, Baccino FM and Sargus MJ, 1977b, Biochem. J., 168, 223.

6. Amenta JS, Baccino FM and Sargus MJ, 1976, Biochim. Biophys. Acta, 451, 511.

7. Amenta JS, Baccino FM and Sargus MJ, 1978b, J. Cell Physiol., 97, 267.

8. Amenta JS and Brocher SC, 1981, Life Sci., 28, 1195.

9. Amenta JS and Brocher SC, 1980a, J. Cell. Physiol., 102, 259.

10. Amenta JS and Brocher SC, 1980b, Exp. Cell Res., 126, 167.

11. Amenta JS, Hlivko TJ, Mcbee AG, Shinozuka H and Brocher S, 1978a, Exp. Cell Res., 115, 357.

12. Amsterdam A, Nimrod A, Lampfecht SA, Burnstein Y and Lindner HR, 1979, Am. J. Physiol., 236, E129.

13. Anderson RGW, Brown MS, Beisiegel U and Goldstein JL, 1982, J. Cell Biol. 93, 523.

14. Anderson LL, Collins GJ, Ojima Y and Sullivan RD, 1970, Cancer Res., 30, 1344.

15. Arnold Jr. LJ, Dagan A and Kaplan NO, 1983 in: Targeted Drugs, ed. Goldberg EP (John Wiley and Sons, Inc., New York) 2, 1.

16. Auricchio F, Martin Jr. D and Tomkins G, 1969, Nature, 224, 806.

17. Auteri JS, Okada A, Bochaki V and Dice JF, 1983, J. Cell Physiol., 115, 167.

18. Avrameas S, Ternynck T and Guesdon JL, 1978, Scand. J. Immunol., 8, Suppl. 7, 7.

19. Axline SG and Cohn ZA, 1970, J. Exp. Med. 131, 1239.

20. Ayusawa D, Koyama H and Seno T, 1981, Cancer Res., 41, 1497.

21. Baggott, JE, 1983, Fed. Proc., 42, 667 abstract.

22. Baker BR, 1959, Cancer Chemother. Rep., 4, 1.

23. Baker, BR and Ho BT, 1964, J. Pharm. Sci., 53, 1457.

24. Balinska M, Galivan J and Coward JK, 1981, Cancer Res., 41, 2751.

25. Baugh CM, Krumdieck CL and Nair MG, 1973, Biochem. Biophys. Res. Commun., 52, 27.

26. Bender RA, 1979, in: High Dose Methotrexate, Pharmacology, Toxicology and Chemotherapy, ed. Periti P (Firenze, Editrice Giuntina) p. 23.

27. Bennett HS, 1956, J. Biophys. Biochem. Cytol. Suppl. 2, 99.

28. Bennet CD, Rodkey JA, Sondey JM and Hirschmann R, 1978, Biochemistry 11, 1328.

29. Bertino JR, 1975, in: Antineoplastic and Immunosuppressive Agents II, eds. Sartorelli AC and Johns DG (Springer, New York)

p. 468.

30. Bertino JR, Booth BA, Bieber AL, Cashmore A and Sartorelli AC, 1964, J. Biol. Chem., 239, 479.

31. Bertino JR, Sawicki WL, Cashmore AR, Cadman EC and Skeel RT, 1977, Cancer Treat. Rep. 61, 667.

32. Bischoff KB, Dedrick RL, Zaharko DS and Longstreth JA, 1971, J. Pharm. Sci., 60, 1128.

33. Bitar KG, Blakenship DT, Walsh KA, Dunlap RB, Reddy AV and Freisheim JH, 1977, FEBBS Lett., 80, 119.

34. Blakely RL, 1969, The Biochemistry of Folic Acid and Related Pteridines (North-Holland, Amsterdam) Chap. 5,6,7,8 and 9.

35. Bleyer WA, 1978, Cancer, 41, 36.

36. Blume KG, Bentler E, Bross KG et al., 1980, N. Engl. J. Med., 302, 1041

37. Bowers B and Olszewski TE, 1972, J. Cell Biol., 53, 681

38. Brown MS, Anderson RGW and Goldstein JL, 1983, Cell, 32, 663

39. Burstein S and Knapp R, 1977, J. Med. Chem., 20, 950.

40. Burtin P, Hartmann L, Fauvert R and Grabar P, 1956, Rev. Fr. Etud. Clin. Biol., 1, 17.

41. Calvert AH, Bondy PK and Harrap KR, 1977, Cancer Treat. Rep., 61, 1647

42. Chabner B, ed., 1982, The Pharmacologic Principles of Cancer Treatment (WB Saunders, Philadelphia) p. 229

43. Chabner BA and Young RC, 1973, J. Clin. Invest., 52, 1804

44. Charlton PA and Young DW, 1979, Chem. Commun., 922.

45. Chu BCF, Fan CC and Howell SB, 1981, JNCI, 66, 121

46. Chu BCF and Whiteley JM, 1977, Mol. Pharmacol., 12, 80

47. Chu BCF and Whiteley JM, 1980, Mol. Pharmacol., 17, 382

48. Chungi VS, Bourne DWA and Dittert LW, 1978, J. Pharm. Sci., 67, 560

49. Clendeninn NJ, Cowan KH, Kaufman BT, Nadkarni MV and Chabner BA, 1983, Proc. Am. Assoc. Cancer Res., 24, 276 abstract

50. Cohen LA, 1974, Methods Enzymol., 34, 102

51. Comaish S and Juhlin L, 1969, Arch. Derm., 100, 99

52. Cohn ZA, 1966, J. Exp. Med., 124, 557

53. Cohn ZA, Fedorko ME and Hirsch JG, 1966, J. Exp. Med., 123, 757

54. Courtoy PJ, Quintart J, Limet JN, De Rbe C and Baudhuin P, 1982, J. Cell Biol., 95, 425a

55. Crowther RA and Pearse BMF, 1981, J. Cell Biol., 91, 790

56. DeCarvalho S, Rand HJ and Lewis A, 1964, Nature, 202, 255

57. Dedrick RL, Zaharko DS, Bender RA, Bleyer WA and Lutz RJ, 1975, Cancer Chemother. Rep., 59, 795

58. Dedrick RL, Zaharko DS and Lutz RJ, 1973, J. Pharm. Sci., 62, 882

59. de Duve C, 1983, Eur. J. Biochem., 137, 391

60. de Duve C and Wattiaux R, 1966, Annu. Rev. Physiol., 28, 435

61. Dembo M and Sirotnak FM, 1976, Biochim. Biophys. Acta, 448, 505

62. Dembo M, Sirotnak FM and Moccio DM, 1984, J. Membrane Biol., 78, 9

63. Deutsch H, Kratochvil C and Reif A, 1955, J. Biol. Chem., 216, 103

64. Dickson RB, Willingham MC and Pastan IH, 1981, J. Biol. Chem., 256, 3454

65. Donehower RC, Hande KR, Drake JC and Chabner BA, 1979, Clin. Pharmacol. Ther., 26, 63

66. Droller MJ and Roth TF, 1966, J. Cell Biol. 28, 209

67. Edelman GM, 1970, Biochemistry, 9, 3197

68. Edelman GM, 1973, Science, 180, 830

69. Edelman GM and Poulik MD, 1961, J. Exp. Med., 113, 861

70. Edelson PJ and Cohn ZA, 1974, J. Exp. Med., 140; 1364

71. Edwards DC, 1983, Pharmac. Ther., 23, 147

72. Ehrlich P, 1957, The Collected Papers of Paul Ehrlich (Pergamon, London) 2, 550

73. Engers HD and Unanue ER, 1973, J. Immunol., 110, 465

74. Evans WE and Pratt CB, 1978, Clin. Pharmacol. Ther., 23, 68

75. Fabre G, Matherley LH, Fabre I, Cano J-P and Goldman ID, 1984, Cancer Res., 44, 970

76. Farber S, Diamond LK, Mercer RD, Silverster Jr. RF and Wolff JA, 1948, N. Engl. J. Med., 228, 787

77. Farquhar MG and Palade GE, 1960, J. Biophys. Biochem. Cytol., 7, 297

78. Fawcett DW, 1964, J. Histochem. Cytochem., 13, 75

79. Fedorko ME, Hirsch JG and Cohn ZA, 1968, J. Cell Biol., 38, 392

80. Fischer GA, 1962, Biochem. Pharmacol., 11, 1233

81. Fish, F, Witz IP and Klein G, 1974, Clin. Exp. Immunol., 16, 355

82. Fisher RI, Chabner BA and Myers CE, 1979, Cancer Treat. Rep., 63,

47

83. Fleischman JB, Pain RH and Porter RR, 1962, Arch. Biochem. Biophys., 99 Suppl 1, 174

84. Flintoff WF and Essani K, 1980, Biochemistry, 19, 4321

85. Foley EJ, 1953, Cancer Res., 13, 835

86. Friend DS and Farquhar MG, 1967, J. Cell Biol., 35, 357

87. Froese G, Linford JH, Berczi I, Sehon AH and Israels LG, 1976, Proc. Am. Assoc. Cancer Res., 17, 120 abstract

88. Fry DW, Anderson LA, Borst M and Goldman ID, 1983, Cancer Res., 43, 1087.

89. Fry DW, White JC and Goldman ID, 1980, Cancer Res., 40, 3669

90. Fry DW, Yalowich JC and Goldman ID, 1982, J. Biol. Chem., 257, 1890

91. Fukumoto T and Brandon MR, 1981, Mol. Immunol., 18, 741

92. Futterman S, 1957, J. Biol. Chem., 228, 1031

93 Fyfe MJ and Goldman ID, 1973, J. Biol. Chem., 248, 5067

94 Galivan J, 1980, Mol. Pharmacol., 17, 105

95 Galivan J, Balinska M and Whiteley JM, 1982, Arch. Biochem. Biophys., 216, 544

96 Galloway CJ, Dean GE, Marsh M, Rudnick G, Mellman I, 1983, Proc. Natl. Acad. Sci., USA, 80, 3334

97 Gaukroger J, Wilson L, Stewart M, Farid Y, Habeshaw T, Harding N, Mackie R, 1983, Br. J. Cancer, 47, 671

98 Geisow MJ, Hart PD and Young MR, 1981, J. Cell Biol., 89, 645

99. Gergely J, Medgyesi GA, Wang AC and Fudenberg HH, 1972,

Immunochemistry 9, 589

100 Geuze HJ, Slot JW, Strous GJAM, Lodish HF and Schwartz AL, 1982, J. Cell Biol., 92, 865

101 Gewirtz DA, White JC, Randolph JK and Goldman ID, 1980, Cancer Res., 40, 573

102 Gewirtz DA, White JC, Randolph JK and Goldman ID, 1979, Cancer Res., 39, 2914

103 Ghetie V and Motas C, 1971, Immunochemistry, 8, 89

104 Ghose T and Blair AH, 1978, J. Natl. Cancer Inst., 61, 657

105 Ghose T, Ferrone S, Imai K, Norvell Jr. ST, Luner SJ, Martin RH and Blair AH, 1982, JNCI, 69, 823

106 Ghose T, Guclu A, Tai J, Mammen M and Norvell Jr, ST, 1977, Eur. J. Cancer, 13, 925

107 Ghose T, Kulkarni PN, Ferrone S, Blair AH, Mammen M and Sadi D, 1984, Cancer Res. Submitted for Publication

108 Ghose T, Nairn RC and Fothergill JE, 1962, Nature, 196, 1108

109 Ghose T and Nigam SP, 1972, Cancer, 29, 1398

110 Ghose T, Norvell Jr. ST, Guclu A and MacDonald AS, 1975, Eur. J. Cancer, 11, 321

111 Ghose T, Norvell Jr. ST, Guclu A et al., 1972, Brit. Med. J., 3, 495

112 Ghose, T, Tai J, Guclu A, Norvell, Jr. ST, Bodurtha A, Aquino J and McDonald AS, 1976, Ann. NY. Acad. Sci., 277, 671

113 Gilliland DG and Collier RJ, 1980, Cancer Res., 40, 3564

114 Gleisner JM, Peterson DL and Blakely RL, 1974, Proc. Natl. Acad.

Sci., USA, 71, 3001

115 Glimelius B, Westermark KB and Wasteson A, 1977, Exp. Cell Res., 108, 23

116 Goldie JH, Krystal G, Hartley D, Gudanskas G and Dedhar S, 1980, Eur. J. Cancer, 16, 1539

117 Goldman ID, 1974, Mol. Pharmacol., 10, 257

118 Goldman ID, 1975, Cancer Chemother. Rep., 6, 51

119 Goldman ID, 1977, Cancer Treat. Rep., 61, 549

120 Goldman ID, 1971, Ann. NY. Acad. Sci., 186, 400

121 Goldman ID, 1969, J. Biol. Chem., 244, 3779

122 Goldman ID, 1982, in: Pharmacologic Principles of Cancer Treatment. ed. Chabner B (WB Saunders, Philadelphia) p.15

123. Goldman ID, Lichtenstein NS and Oliverio VT, 1968, J. Biol. Chem., 243, 5007

124 Goldstein JL, Anderson RGW and Brown MS, 1979, Nature, 279, 679

125 Goldstein JL, Anderson RGW and Brown MS, 1982, Membrane Recycling Ciba Found. Symp., 92, 77

126 Goldstein JL, Basu SK, Brunschede GY and Brown MS, 1976, Cell 7, 85

127 Gonatas NK, Gonatas JD, Stieber A, Antoine JC, Avrameas S, 1976, J. Cell Biol., 70, 477

128 Gordon GB, Miller LR and Bensch KG, 1965, J. Cell Biol., 25, pt 2, 41

129 Graham RC Jr. and Karnovsky MJ, 1966, J. Histochem. Cytochem., 14, 291

130 Gregoriadis G, 1977, Nature, 265, 407

131 Gross L, 1974, Cancer Res., 3, 326

132 Guclu A, 1975, Ph.D Thesis, Dalhousie University

133 Guclu A, Tai J and Ghose T, 1975, Immunol. Commun., 4, 229

134 Gunn JM, 1978, Exp. Cell Res., 117, 448

135 Gutterman JU and Hersch EM, 1982, in: Cancer Medicine, eds. Holland JF and Frei E III (Lea and Febiger, Philadelphia) p.1100

136 Haber DA, Beverly SM, Kiely ML and Schimke RT, 1981, J. Biol. Chem., 256, 9501

137 Haigler HT, Maxfield FR, Willingham MC and Pastan I, 1980, J. Biol. Chem., 255, 1239

138 Hanggi UJ and Littlefield JW, 1974, J. Biol. Chem., 249, 1390

139 Harding NGL, Martelli MF and Huennekens FM, 1970, Arch. Biochem. Biophys., 137, 295

140 Henderson ES, Adamson RH, Denham C and Oliverio VT, 1965a, Cancer Res., 25, 1008

141 Henderson ES, Adamson RH and Oliverio VT, 1965b, Cancer Res., 25, 1018

142 Henderson GB and Zevely EM, 1980, Arch. Biochem. Biophys., 200, 149

143 Henderson GB and Zevely EM, 1982, Biochem. Biophys. Res. Commun., 104, 474

144 Henderson GB and Zevely EM, 1984, J. Biol. Chem., 259, 1526

145 Henderson GB, Zevely EM and Huennekens FM, 1980, J. Biol. Chem., 255, 4829

146 Hershko A and Ciechanover A, 1982, Annu. Rev. Biochem., 51, 335

147 Hershko A and Tomkins GM, 1971, J. Biol. Chem., 246, 710

148 Hill BT, Bailey BD, White JC and Goldman ID, 1979, Cancer Res., 39, 2440

149 Hill RL, Delaney R, Lebovitz HE and Fellows RE, 1966, Proc. Roy. Soc. Ser B, 166, 159

150 Hoffer PB and Gottschalk A, 1976, Semin. Nucl. Med., 4, 305

151 Hood K and Roberts GCK, 1978, Biochem. J., 171, 357

152 Huennekens FM, 1968, in: Biological Oxidations, ed. Singer TP (Wiley, New York) p.439

153 Huennekens FM and Scrimgeour KG, 1964, in: Pteridine Chemistry eds. Pfleiderer W and Taylor EC (Pergamon, New York) p. 355

154 Huffman DH, Wan SH, Azarnoff DL and Hoogstraten B, 1973, Clin. Pharmacol. Ther., 14, 572

155 Hurwitz E, Levy R, Maron R, Wilchek M, Arnon R and Sela M, 1975, Cancer Res., 35, 1175

156 Imai K, Ng A-K and Ferrone S, 1981, JNCI, 66, 489

157 Jackson RC and Harrap KR, 1973, Arch. Biochem. Biophys., 158, 827

158 Jackson RC and Niethammer D, 1977, Eur. J. Cancer, 13, 567

159 Jacobs SA, Adamson RH, Chabner BA, Derr CJ and Johns DG, 1975, Biochem. Biophys. Res. Commun., 63, 692

160 Jacobs SA, Derr CJ and Johns DG, 1977a, Biochem. Pharmacol., 26, 2310

161 Jacobs SA, Stoller RG, Chabner BA and Johns DG, 1976, J. Clin. Invest., 57, 534

162 Jacobs SA, Stoller RG, Chabner BA and Johns DG, 1977b, Cancer Treat. Rep., 61, 651

163 Johnson LF, Fuhrman CL and Abelson HT, 1978, Cancer Res., 38, 2408

164 Jolivet J and Chabner BA, 1983, J. Clin Invest., 72, 773

165 Jolivet J, Cowan KH, Curt GA, Glendenium NJ and Chabner BA, 1983, N. Engl. J. Med., 309, 1094

166 Jolivet J, Schilsky RL, Bailey BD and Chabner BA, 1982a, Ann. NY. Acad. Sci., 397, 184

167 Jolivet J, Schilsky RL, Bailey BD, Drake JC and Chabner BA, 1982b, J. Clin. Invest., 70, 351

168 Karakousis CP and Carlson M, 1979, Cancer Treat. Rep., 63, 1405

169 Keen JH, Willingham MC and Pastan IH, 1979, Cell 16, 303

170 Kehoe JM and Fougereau M, 1969, Nature 224, 1212

171 Keisari Y and Witz IP, 1978, JNCI, 61, 1978

172 Keisari Y and Witz IP, 1979, Haematol. Bluttransfus., 23, 407

173 Keisari Y and Witz IP, 1973, Immunochemistry, 10, 565

174 Kessel D, Hall T and Roberts D, 1965, Science, 150, 752

175 Khorana H, 1953, Chem. Rev., 53, 145

176 King AC and Cuatrecasas P, 1981, N. Engl. J. Med., 305, 77

177 Kirschhansen T and Harrison SC, 1981, Cell 23, 755

178 Klein G, 1975, in: Immunological Aspects of Neoplasia (Williams and Wilkins, Baltimore) p.21

179 Knowles SE and Ballard FJ, 1976, Biochem. J, 156, 609

180 Korngold L and Lipari R, 1956, Cancer, 262, 9

181 Krakower GR, Nylen PA and Kamen BA, 1982, Anal. Biochem., 122, 412

182 Kufe DW, Wick MM and Abelson HT, 1980, J. Invest. Dermatol., 75, 357

183 Kulkarni PN, Blair AH and Ghose TI, 1981, Cancer Res., 41, 2700

184 Laemmli UK, 1970, Nature, 227, 680

185 Landsteiner K, 1946, The Specificity of Serological Reactions (Harvard University Press, Cambridge, MA)

186 Lankelma J, van der Kleijn E and Ramaekers F, 1980, Cancer Lett., 9, 133

187 Lankelma J, van der Kleijn E and Termond EFS, 1978, in: Clinical Pharmacology of Antineoplastic Drugs ed. Pinedo HM (Elsevier/North Holland Biomedical Press, Amsterdam) p.13

188 Latif ZA, Lozzio BB, Wust CJ, Krauss S, Aggio MC and Lozzio CB, 1980, Cancer Res., 45, 1326

189 Levitt M, Mosher MS and DeConti RC, 1973, Cancer Res., 33, 1729

190 Lewis WH, 1931, Bull. Johns Hopkins Hosp., 49, 17

191 Leigler DG, Henderson ES, Hahn MA and Oliverio VT, 1969, Clin. Pharmacol. Therap., 10, 849

192 Lineweaver H and Burk D, 1934, J. Am. Chem. Soc., 56, 658

193 La Spalluto J, Fehr K and Ziff M, 1971 in: Tissue Proteinases ed. Barrett AJ and Dingle JT (North Holland, Amsterdam) p. 263

194 Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, 1951, J. Biol. Chem., 193, 265

195 Mathe G, Loc T and Bernard J, 1958, C.R. Acad. Sci., 246, 1626

196 Matthews DA, Alden RA, Bolin JT et al., 1977, Science, 197, 452

197 Matthews DA, Alden RA, Bolin JT et al., 1978, J. Biol. Chem., 253, 6949

198 Matthews CK and Huennekens FM, 1963 J. Biol. Chem., 238, 3436

199 Maver ME, Dunn TB and Greco A, 1945, J. Natl. Cancer Inst., 6, 69

200 Maver ME, Dunn TB and Greco A, 1948, J. Natl. Cancer Inst., 9, 39

201 Maxfield FR, 1982, J. Cell Biol., 95, 676

202 Maxfield FR, Schlessinger J, Schechter Y, Pastan I and Willingham MC, 1978, Cell, 14, 805

203 McGurre JJ, Hsieh P, Coward JK and Bertino JR, 1980, J. Biol. Chem., 255, 5776

204 Melera PW, Lewis JA, Biedler JA and Hession C, 1980, J. Biol. Chem., 255, 7024

205 Mellman IS, 1982, Membrane Recycling Ciba Found. Symp. 92, 35

206 Merisko EM, Farquhar MG and Palade GE, 1982, J. Cell Biol., 92, 846

207 Moolten FL, Capparell NJ, Zajdel SH and Cooperband SR, 1975, J. Natl. Cancer Inst., 55, 473

208 Moolten F, Zajdel S and Cooperband S, 1976, Ann. NY Acad. Sci., 277, 690

209 Moran RG, Mulkins M and Heidelberger C, 1979, Proc. Natl. Acad. Sci. USA, 76, 5924.

210 Motas C and Ghetie V, 1969, Immunochemistry, 6, 547

211 Neff DT, De Martino GM and Goldberg AL, 1979, J. Cell Physiol., 101, 439

212 Nisonoff A, Wissler FC, Lipman LN and Woernley DL, 1960, Arch. Biochem. Biophys., 89, 230

213 Odor DL, 1956, J. Biophys. Biochem. Cytol. Suppl. 2, 105

214 Ohkuma S, Moriyama Y and Takano T, 1982, Proc. Natl. Acad. Sci., USA, 79, 2758

215 Ohkuma S and Poole B, 1978, Proc. Natl. Acad. Sci., USA, 75, 3327

216 Olsnes S and Pihl A, 1982, Pharmac. Ther., 15, 355

217 Osborn MJ, Freeman M and Huemmekens FM, 1958, Proc. Soc. Exptl. Biol. Med., 97, 429

218 Palade GE, 1956, J. Biophys. Biochem. Cytol Suppl. 2, 85

219 Palade GE, 1982, Membrane Recycling Ciba Found. Symp., 92, 293

220 Pastan IR and Willingham MC, 1981, Science, 214, 504

221 Pearse BMF, 1976, Proc. Natl. Acad. Sci., USA, 73, 1255

222 Pearse BMF and Bretscher MS, 1981, Annu. Rev. Biochem., 50, 85

223 Peterson DL, Gleisner JM and Blakely RL, 1975a J. Biol. Chem., 250, 4945

224 Peterson DL, Gleisner JM and Blakely RL, 1975b, Biochemistry, 14, 5261

225 Pinedo HM and Chabner BA, 1977, Cancer Treat. Rep., 61, 709

226 Pinedo HM, Zaharko DS, Bull J and Chabner BA, 1977, Cancer Res., 37, 445

227 Pitman SW, Parker LM, Tattersall MHN, Jaffe N and Frei E III, 1975, Cancer Chemother. Rep., 6, 43

228 Poole B, Ohkuma S and Warburton M, 1978, in: Protein Turnover and Lysosomal Function eds. Segal HL and Doyle DJ (Academic

Press, New York) p. 43

229 Poole B and Wibo M, 1973, J. Biol. Chem., 248, 6221

230 Porter RR, 1959, Biochem. J, 73, 119

231 Posner BI, Bergeron JJM, Josefsberg Z et al., 1981, Recent Prog. Hormone Res., 37, 539

232 Poser RG, Sirotnak, FM and Chello PL, 1980, Biochem. Pharmacol., 29, 2701

233 Poser RG, Sirotnak FM and Chello PL, 1981, Cancer Res., 41, 4441

234 Purr A, 1934, Biochem. J., 28, 1907

235 Radar JI and Huennekens FM, 1973, in: The Enzymes ed. Paul Boyer (Academic Press, New York) 9, 197

236 Radar J, Niethammer D and Huennekens FM, 1974, Biochem. Pharmacol., 23, 2057

237 Regoeczi E, Chindemi PA, DeBanne MT, Charlwood PA, 1982, Proc. Natl. Acad. Sci., USA, 79, 2226

238 Rez G, Kiss A, Bucsek MJ and Kovacs J, 1976, Chem. Biol. Inter., 13, 77

239 Robinson DA, Whiteley JM and Harding NG, 1973, Biochem. Soc. Trans., 1, 722

240 Rosenblatt DA, Whitehead VM, Dupont MM, Vuchich M-J and Vera N, 1978a, Mol. Pharmacol., 14, 210

241 Rosenblatt DS, Whitehead VM, Vera N, Pottier A, Dupont M and Vuchich M-J, 1978b, Mol. Pharmacol., 14, 1143

242 Rosenblatt DS, Whitehead VM, Vuchich M-J, Pottier A, Matiaszuk UV and Beaulieu B, 1981, Mol. Pharmacol., 19, 87

243. Rosowsky A, Forsch RA, Freisheim JF, Galivan J and Wick M, 1984, J. Med. Chem., 27, 888

244 Roth TF and Porter KR, 1964, J. Cell Biol., 20, 313

245 Rubens Rd, 1974, Lancet, 1, 498

246 Ryser HJ-P, 1968, Science, 159, 390

247. Schersten T, Wahlqvist L and Jilderos B, 1971, Cancer, 27, 278

248 Schilsky RL, Bailey RD and Chabner BA, 1980, Proc. Natl. Acad. Sci., USA, 77, 2919

249 Schimke RT, 1980, Sci. Am., 243, 50

250 Schneider DL, 1981, J. Biol. Chem., 256, 3858

251 Schneider Y-J, de Duve C and Trouet A, 1981, J. Cell Biol., 88, 380

252 Schornagel JH and McVie JG, 1983, Cancer Treat. Rev., 10, 53

253 Schwartz AL, Fridovich SE and Lodish HF, 1982, J. Biol. Chem., 257, 4230

254 Seglen PO, 1983, Methods Enzymol., 96, 737

255 Seglen PO, 1975, Biochem. Biophys. Res. Commun., 66, 44

256 Seglen PO, 1976, in: Use of Isolated Liver cells and Kidney Tubules in Metabolic Studies eds. Tager JM, Soling ND and Williamson JR (North-Holland, Amsterdam) p.245

257 Seglen PO, 1977, Biochim. Biophys. Acta, 496, 182

258 Seglen PO and Reith A, 1976, Exp. Cell Res., 100, 276

259 Shamberger RJ, Hozumi M and Morris HP, 1971, Cancer Res., 31, 1632

260 Sheehan J and Hess G, 1955, J. Amer. Chem. Soc., 77, 1067

261 Shen W-C and Ryser HJ-P, 1984, Proc. Natl Acad. Sci., USA, 81, 1445

262 Shen W-C and Ryser HJ-P, 1979, Mol. Pharmacol., 16, 614

263 Silber R, Huennekens FM and Gabrio BW, 1963, Arch. Biochem. Biophys., 100, 525

264 Silverstein SC, Steinman RM and Cohn ZA, 1977, Annu. Rev. Biochem., 46, 669

265 Sirotnak FM, 1980, Pharmac. Ther., 8, 71

266 Sirotnak FM and Donsbach RC, 1976, Cancer Res., 36, 1151

267 Sirotnak FM, Moccio DM, Goutas LJ and Kelleher LE, 1982, Cancer Res., 42, 924

268. Sirotnak FM, Moccio DM and Young CW, 1981a Cancer Res., 41, 966

269 Sirotnak FM, Moccio DM, Kelleher LE and Goutas LJ, 1981b, Cancer Res., 41, 4447

270 Sklar LA, Jesaitis AJ, Painter RG and Cochrane CG, 1982, J. Cell Biochem., 20, 193

271 Sobczak E and de Vaux Saint Cyr Ch., 1971, Int. J. Cancer, 8, 47

272 Steele WH, Stuart JB, Lawrence JR and Calman KC, 1979a, Br. J. Cancer, 40, 316

273 Steele WH, Lawrence JR, Stuart JFB and McNeill CA, 1979b, Eur. J. Clin. Pharmacol., 15, 363

274 Steele WH, Stuart JFB, Lawrence JR and McNeill CA, 1982, Cancer Chemother. Pharmacol., 9, 110

275 Steinman RM, 1976, in: In vitro Methods in Cell Mediated and Tumor Immunity eds. Bloom BR and David JR (Academic Press, New York) p.379

276 Steinman RM, Brodie SE and Cohn ZA, 1972, J. Cell Biol., 68, 665

277 Steinman RM and Cohn ZA, 1972 J. Cell Biol., 55, 186

278 Steinman RM, Mellman IR, Muller WA and Cohn ZA 1983, J. Cell Biol., 96, 1

279 Steinman RM, Silver JM and Cohn ZA, 1974, J. Cell Biol., 63, 949

280 Stoller RG, Jacobs SA, Drake JC, Lutz RJ and Chabner BA, 1975, Cancer Chemother. Rep., 6, 19

281 Stone D, Paterson SJ, Raper JH and Phillips AW, 1979, J. Biol. Chem., 254, 480

282 Straus W, 1964, J. Cell Biol., 20, 497

283 Stuart JFB, Calman KC, Watters J et al., 1979, Cancer Chemother. Pharmacol., 3, 239

284 Sylven B, 1968, Eur. J. Cancer, 4, 463

285 Sylven B and Bois-Svensson I, 1965, Cancer Res., 25, 458

286 Sullivan AL, Grasso JA and Weintraub LR, 1976, Blood, 47, 133

287 Szeto DW, Cheng Y, Rosowsky A et al., 1979, Biochem. Pharmacol., 28, 2633

288 Tai J, Blair AH and Ghose T, 1976, Proc. Can. Fed. Biol. Sci., 19, 502 abstract

289 Taylor RB, Duffus WPH, Raff MC and De Petris S, 1971, Nature New Biol., 233, 225

290 Tolleshaug H, 1981, Int. J. Biochem., 13, 45

291 Tycko B and Maxfield FR, 1982, Cell, 28, 643

292 Unanue ER, Karnovsky MJ and Engers HD, 1973, J. Exp. Med., 137, 675

293 Unanue ER, Perkins WD and Karnovsky MJ, 1972, J. Exp. Med., 136, 885

294 Unanue ER, Ungewickell E and Branton D, 1981, Cell, 26, 439

295 Ungewickell E and Branton D, 1981, Nature, 289, 420

296 Valentine RC and Green NM, 1967, J. Mol. Biol., 27, 615

297 Valerino DM, Johns DG, Zaharko DS and Oliverio VT, 1972, Biochem. Pharmacol., 21, 821

298 Verella G and Parkhouse RME, 1971, Immunochemistry, 8, 243

299 Wagner R, Rosenberg M and Estensen R, 1971, J. Cell Biol., 50, 804

300 Wall DA, Wilson G and Hubbard AL, 1980, Cell, 21, 79

301 Wan SH, Huffman DH, Azarnoff DL, Stephens R and Hoogstraten B, 1974, Cancer Res., 34, 3487

302 Warren RD, Nichols AP and Bender RA, 1978, Cancer Res., 38, 668

303 Weigel PH and Oka JA, 1984, J. Biol. Chem., 259, 1150

304 Weinstein GD, 1977, Ann. Intern. Med., 86, 199

305 Weitzman SA, Desmond MC and Stossel TP, 1979, J. Clin. Invest., 64, 321

306 Werkheiser WC, 1961, J. Biol. Chem., 236, 888

307 Weygand F, Hoffman D and Wunsch E, 1966, Z. Naturforsch Teil B, 21, 426

308 Whitehead VM, 1977, Cancer Res., 37, 408

309 Whitehead VM, Perrault MM and Stelcner S, 1975, Cancer Res., 35, 2985

310 Wibo M and Poole B, 1974, J. Cell Biol., 63, 430

311 Williams KE; Kidston EM, Beck F, Lloyd JB, 1975, J. Cell Biol., 64, 113

312 Willingham MC and Pastan IH, 1982, J. Cell Biol., 94, 207

313 Willingham MC and Pastan IH, 1983, in: Receptor Mediated Endocytosis eds. Cuatrecasas P and Roth TF (Chapman and Hall, London), 15, 1

314 Willkens RF and Watson MA, 1982, J. Lab. Clin. Med., 100, 314

315 Wilson BS, Imai K, Natali PG and Ferrone S, 1981, Int. J. Cancer, 28, 293

316 Witte A, Whitehead VM, Rosenblatt DS and Vuchich M-J, 1980, Dev. Pharmacol. Ther., 1, 40

317 Woodward MP and Roth TF, 1979, J. Supramol. Struct., 11, 237

318 Wunsch E and Drees F, 1966, Chem. Ber., 99, 110

319 Yasmeen D, Ellerson JR, Dorrington KJ and Painter RH, 1976, J. Immunol., 116, 518

320 Young RC and Chabner BA, 1973, J. Clin. Invest., 52, 92

321 Youngdahl-Turner P, Mellman IS, Allen RH and Rosenberg LE, 1979, Exp. Cell Res., 118, 127

322 Zaharko DS and Dedrick RL, 1977, Cancer Treat. Rep., 61, 513

323 Zaharko DS, Dedrick RL and Bischoff KB, 1971, J. Natl. Cancer Inst., 46, 775

324 Zakrzewski SF and Nichol CA, 1958, Biochim. Biophys. Acta, 27, 425