THE CLINICAL PHARMACOLOGY OF METHOTREXATE

A Thesis submitted to the University of Glasgow in Cadidature for the Degree of Doctor of Philosophy in the Faculty of Medicine by

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To

Maggie

C O N T E N T S

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LIST OF TABLES

LIST OF FIGURES

A C K N O W L E D G M E N T S

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SUMMARY

The investigations reported in this thesis were undertaken to examine the clinical pharmacology of methotrexate. The following results have been observed.

1) The radioimmunoassay employed for the measurement of methotrexate in these studies has a low limit of sensitivity and high precision. In addition it is highly specific and does not cross-react significantly with 7-hydroxymethotrexate.

2) Methotrexate was readily detected, soon after administration in mixed and parotid saliva and also in tears. A close linear correlation was observed between methotrexate concentrations in tears and serum, and also between levels in parotid saliva and serum. No significant correlation could be demonstrated between methotrexate concentrations in mixed saliva and serum. The ratio of drug levels in tears to that unbound in serum was close to unity whereas methotrexate appeared to be secluded from saliva secretion. No relationship between tear levels of drug and conjunctivitis could be demonstrated. The prediction of drug levels in serum based on the concentration measured in either tears or parotid saliva was considered impractical.

3) Methotrexate was also readily detected in cerebrospinal fluid (C.S.F.) of children on high dose intravenous therapy. There was a highly significant linear correlation between the concentration in mixed saliva and concentration in C.S.F. The need for further study was indicated since saliva levels of methotrexate may provide a non-invasive and painless means of monitoring C.S.F. levels of this agent.

4) Methotrexate persists in erythrocytes for a long time probably several weeks after administration of high doses by continuous infusion. It is possible that this process may be described by two mechanisms, mainly, an initial rapidly reversible uptake and a second active uptake process. The concentration/time profile is consistent with erythrocytes being a slowly exchanging kinetic compartment. No immediate displacement of methotrexate was effected by folinic acid administration. The uptake of methotrexate may however, be influenced by prior chemotherapy. It is expected that this persistence in erythrocytes may influence the expression of toxicity and/or resistence.

5) Continuous ultrafiltration was shown to be a viable method for the elucidation of serum protein binding of methotrexate. Methotrexate is bound predominantly to serum albumin. In normal healthy volunteers the binding was linear over a wide range of total concentrations and is highly protein bound over the concentrations encountered after convential doses. Non-linear binding to albumin occurs at higher serum concentrations, such as would occur after high dose therapy. Two groups of binding sites were observed, one being a high affinity/low capacity group, and the other a low affinity group, with a higher capacity. No significant difference in percent bound, nor in binding parameter values could be demonstrated between newly diagnosed patients, with various forms of neoplastic disease, in an age and sex matched group when the group values were compared. The serum protein binding of methotrexate is dependent on the concentration of serum albumin.

6) The absorption from the gastro-intestinal tract of methotrexate formulated as a syrup is saturable. The bioavailability can be conviently improved by dividing high doses into units of 25 mg. given at two hourly intervals. This regimen may permit oral administration to be considered as a reasonable alternative to intravenous therapy.

7) Renal elimination is the main route whereby methotrexate is cleared from the body and it is dose dependent. The studies presented here show that while the exact relationship between renal clearance and methotrexate serum levels awaits clarification, the effect of methotrexate saturable renal elimination can become apparent after modest doses administered by bolus intravenous injection.

Only small quantities of methotrexate are excreted into bile. While this amount is small and suggests negligible enterohepatic recycling it is persistent and may contribute to gastrointestinal toxicity which has been observed after intravenous administration.

CHAPTER 1

INTRODUCTION

SECTION 1 : CHEMOTHERAPY

Principles of Cancer Chemotherapy

The basic assumption in cancer chemotherapy is that all malignant cells should be destroyed or neutralised. Chemotherapy alone, however, has failed to cure most human malignancies, since there is no specific molecular target unique to the cancer cell. The general chemotherapeutic approach is aggressive assault on the functional intergrity of tumour molecular biology (Fig 1). However, treatment is limited not by the extent of the disease, in contrast to surgery or radiotherapy, but rather by the total mass (Laster et al., 1969; Schabel Jr. 1968; Shapiro and Fugmann 1957; Skipper et al., 1964). The evidence for this was provided from experiments conducted by Skipper and his co-workers in the mid 1960's (Skipper et al., 1964; Skipper et al., 1965; Skipper, 1968). It was then proposed that the ability to eradicate malignant cells was dependent not only on the dose of the drug employed but also on the number of tumour cells present i.e., the total body cell burden. Further, the cell destruction followed first order kinetics, i.e., a given treatment will destroy a constant fraction of cells and not a fixed number. The obvious implications of this fraction cell kill hypothesis are, that for drug treatment to be effective in obliterating a tumour population, it would be necessary to:-

a) increase the dose of drug or drugs to just within the limit tolerated by the host.

b) start treatment when the number of tumour cells present is small.

Skipper et al., (1965) also emphasised how critical the interval between doses was for effective therapy, since cell kill per treatment was shown to be the initial cell kill less the re-growth of the tumour population between treatments. Although the fraction kill hypothesis was derived from the leukaemia L1210 model system, the implications may be generally applicable, since the hypothesis has been validated in other animal tumours (Wilcox, et al., 1965; Goldin, et al., 1956; De Vita Jr. 1971) and appears to be true in some human disease (De Vita Jr. 1977).

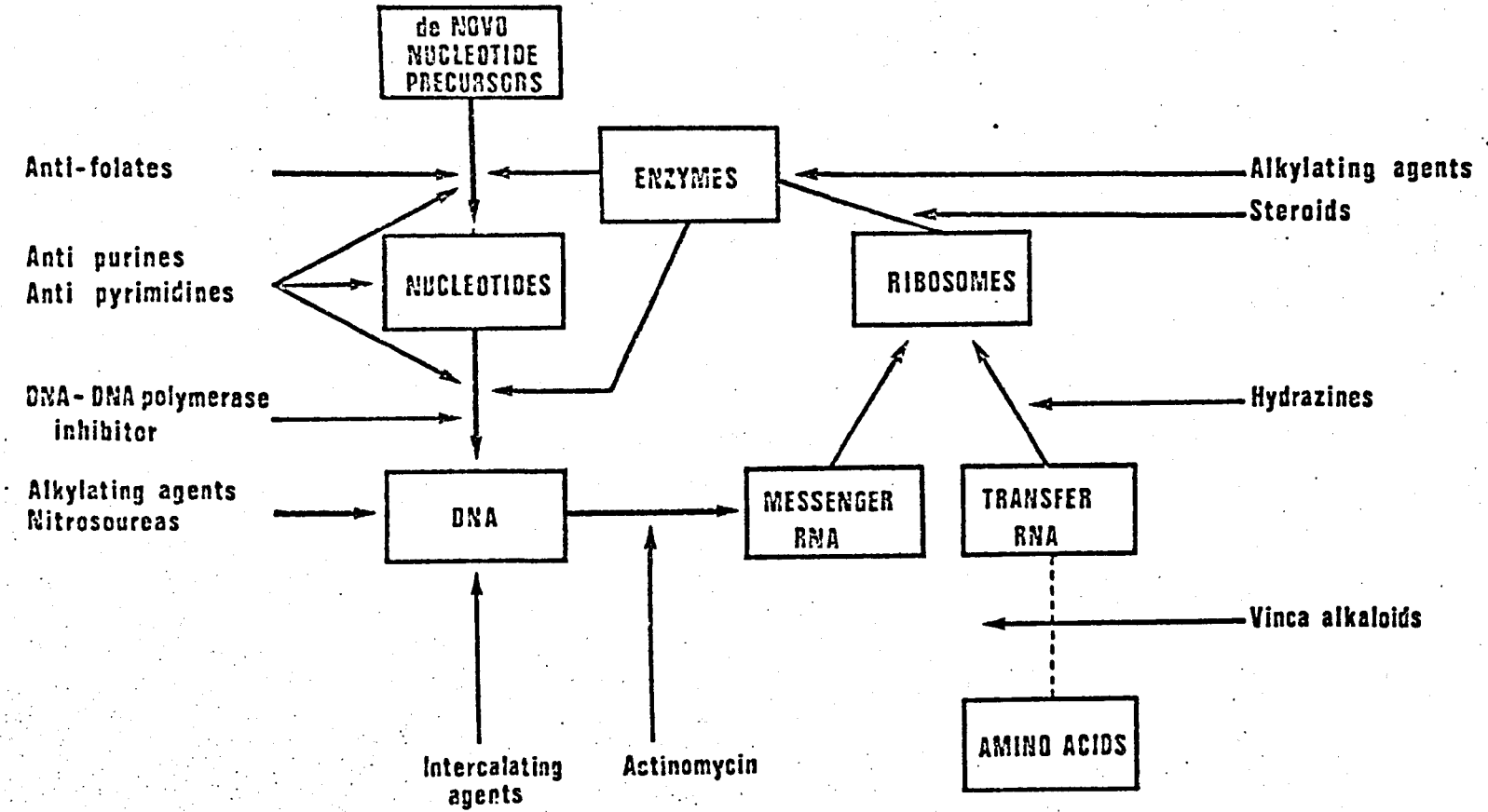


Figure 1 Prime targets of chemotherapeutic assault

The concept that effectiveness of drug treatment increases with decreasing volume of tumour cells was obtained from exponentially growing malignancies. However, most tumours do not have a single growth rate but one that varies continuously with age. Casey (1934) suggested that tumour growth could be described by a Gompertzian curve, although at the time he felt that his data was not firm enough to permit generalisation. It was not until thirty years later that Laird (1964) postulated this mode of growth in solid tumour types. For Gompertzian growth, the rate is least for both very small and very large tumours and is maximum at the inflection point when the tumour is about 37% of its maximum size.

The relationship between tumour size, specific growth and growth rate for unperturbed Gompertzian growth has been well illustrated by Norton and Simon (1977). The work shows clearly the application of the fraction kill hypothesis to this type of growth is unrealistic and the clinical implications which have been drawn from this model are that:-

a) with a very effective therapy a small tumour may be cured by a dose of drug which would not cure a larger tumour.

b) a dose schedule capable of causing a dramatic rate of regression of a tumour of intermediate size may not cure a small tumour when therapy is either reduced or applied over a limited duration.

c) high-dose therapy over a short period may be better than prolonged low-dose treatment in decreasing rates of recurrence.

d) the use of high-doses and perhaps more prolonged therapy (more aggressive therapy) than might otherwise be employed is advocated.

e) intense alternating cycles of different drug combinations in full doses might produce better survival times than using one particular combination of many agents in reduced doses.

It has been proposed by Norton and Simon (1977) that the rate of regression or growth inhibiting effect of therapy is proportional to the growth rate of an untreated tumour.

Knowledge of the process of the cell cycle has also increased the capacity for manipulation of chemotherapeutic agents. The term "cell cycle" describes the period between one mitosis and the next in one or both daughter cells. Howard and Pelc (1951, 1953) first characterised the sequence of events occurring in this cycle by autoradiographic methods. They postulated that the cycle could be divided up, on a temporal basis, into four specific classical phases:-

G_1: the period between mitosis and the onset of DNA synthesis.

S: the period of DNA synthesis.

G_2: the period between completion of DNA synthesis and mitosis.

M: mitosis.

Animal cell populations are, however, heterogeneous and may be divided into three groups (Lajtha, 1963; Baserga, 1971; Hill, 1976; Prescott, 1968). (Fig.2).

1) continuously dividing or cycling cells.

2) resting or non-cycling cells.

3) non-dividing cells.

Most of the variability of cell cycle times for many cells growing under varying or constant conditions arises as a result of the time spent in the G_1 interval, the remaining phases being of relatively fixed duration (Sisken and Kimosita, 1961; Till et al., 1964; Baserga, 1965; Mendelsohn, 1965; Prescott, 1968; Mueller, 1971). Lajtha (1963) introduced the idea that normal cells can move out of the cycle or become arrested for extended periods of time, but still retain the capacity to be stimulated into proliferation. Burns and Tannock (1970) proposed that this variability could be described by assuming that such clonogenic but non-dividing cells enter an intermediate phase, G_0, which is part of G_1. From G_0 they could proceed to the determinate phase 'C' (of constant duration and containing S, G_2, M and G_1 phases), at random with a constant specific rate (γ). Experimental evidence for this theory was obtained from the fraction of cells continuously labelled with (^3H)-thymidine in labelled mitosis in the hamster cheek pouch epithelium.

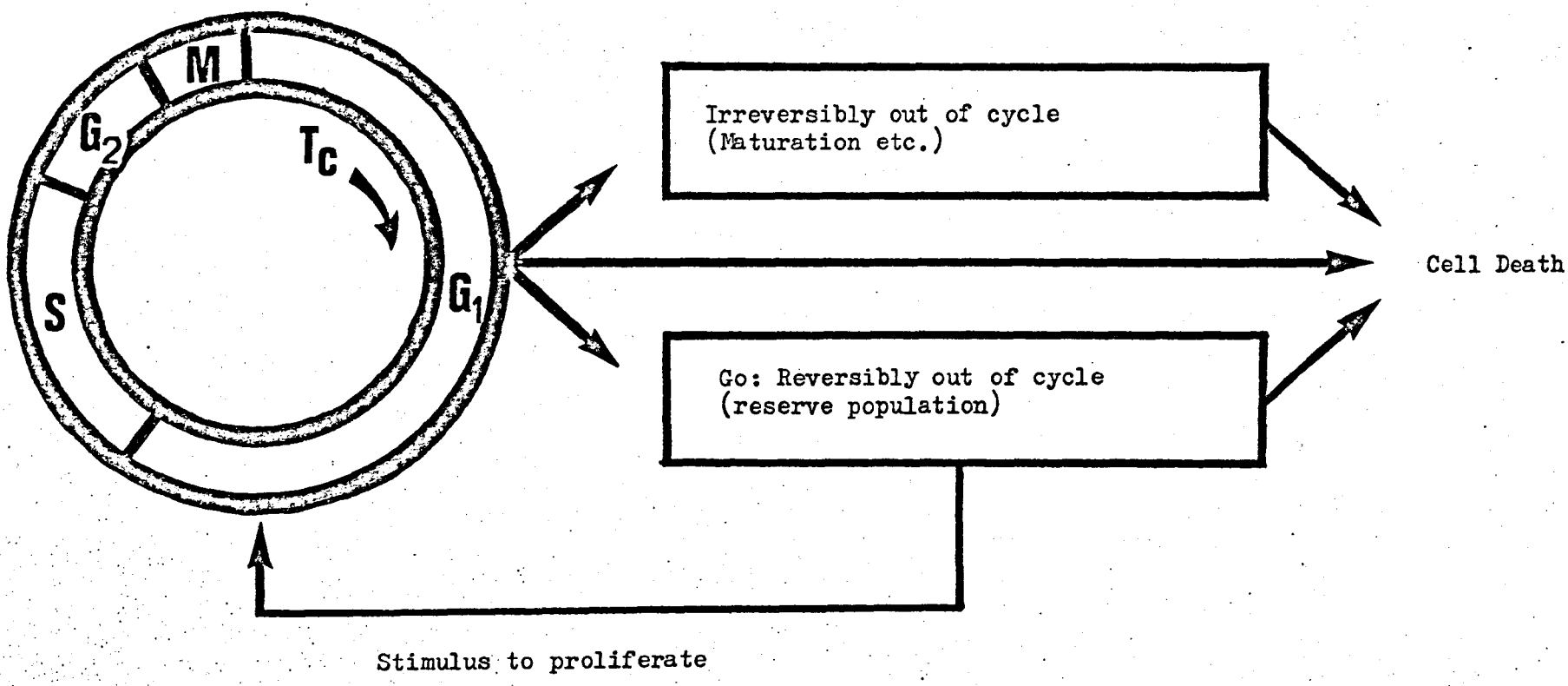


Figure 2 The heterogeneous components of animal cell populations.

Smith and Martin (1973; 1974) later proposed a similar model. According to this model the cycle can be divided into two parts:-

1) The B-phase (equivalent to the 'C' phase) where the cells are proceeding towards division.
2) The A-phase (equivalent to G_0) where the cells are awaiting a random triggering before proceeding.

A cell may remain in the quiescent 'A' state for any length of time without progressing towards mitosis, but it always has a certain chance, called 'transition probability' of entering the B-phase. Brooks (1975; 1976) has observed that the addition of serum to resting fibroblasts increases the transition probability value after a defined lag period. Credibility has been lent to the theory by the shape of frequency of labelled mitosis curves, obtained in vivo, since they can be predicted accurately from the transition probability theory (Burns and Tannock, 1970; Smith and Martin, 1973; De Maertelaer and Galand, 1975; De Maertelaer and Galand, 1977).

Knowledge concerning the cell cycle puts a temporal parameter, albeit a median, on a series of biochemical events which are peculiar to the cell replication function. Since successful chemotherapy depends on a differential action of chemotherapeutic agents on malignant and normal cells, the work of Bruce et al., (1966) was of major importance. They demonstrated a kinetic difference between normal and malignant tissues and indicated how these differences may be exploited to achieve increased selective toxicity of cytotoxic drugs. Further, their work formed the basis for a kinetic classification of anti-cancer drugs which depended on their action during certain phases of the cell cycle and on resting cells. Three broad categories were suggested:-

Class I: Non-specific: the agents appeared to be equally toxic for both proliferating and resting cells.

Class II: Phase-specific: proliferating cells were killed during a specific phase or phases of the cell cycle.

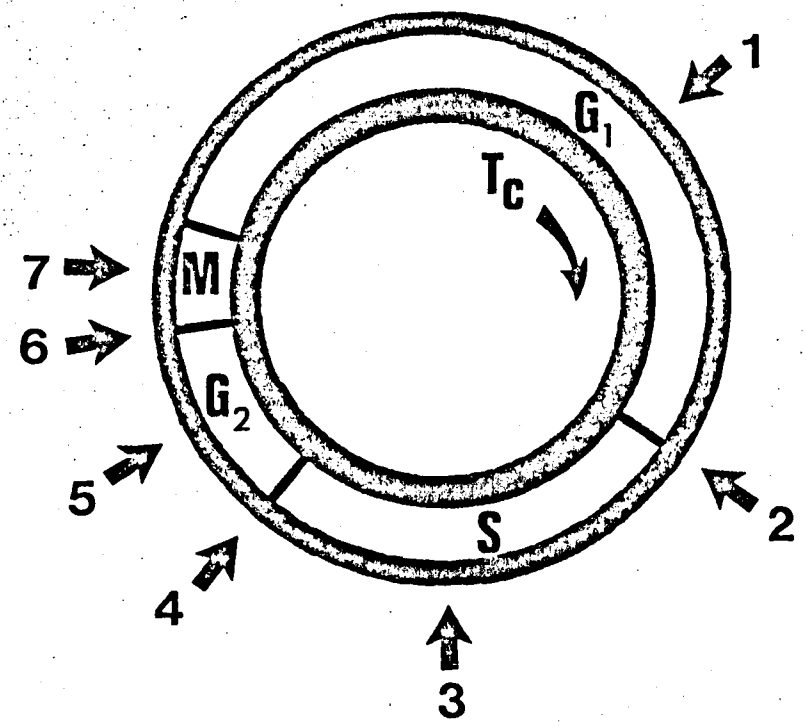
Class III: Cycle-specific: both proliferating and resting cells were damaged, however, dividing cells were more sensitive and were killed throughout the cell cycle.

The specificity of these agents was assessed by the spleen-colony assay developed in mice, in which slowly proliferating haematopoietic and rapidly proliferating mouse lymphoma colony-forming cells were compared. The main implication being a safer cancer chemotherapy involving minimal toxicity to normal bone marrow without compromising antitumour effectiveness. Since this initial work a large number of agents have been classified according to the locus within the cell cycle to which they show greatest activity (Fig. 3).

These studies have provided a rationale for the use of chemotherapeutic agents in the treatment of disseminated disease, the role of adjuvant chemotherapy, and helped in the choice and scheduling of drug combinations. Cure is taken to mean that the life expectancy of the treated patients is the same as that of a matched cohort in the general population.

Although cures or long-term remissions have been obtained in few diseases with chemotherapy alone, extremely encouraging results have been observed in the following conditions: childhood acute lymphoblastic leukaemia has been effectively controlled with chemotherapy alone (Freireich, 1966; Simone, 1974;) Hodgkins disease, which represents one of the more notable advances in the clinical control of cancer (De Vita et al., 1970; De Vita and Schein, 1973; Stutzman and Glidewell, 1973; Frei et al., 1973;) Burkitt's lymphoma (Zeigler, 1973).

It has been obvious for many years that surgical or radiotherapeutic ablation cannot achieve the desired cancer control in a large number of patients. When the tumour is truly localised and can be totally excised or obliterated by radiotherapy, these alone may be sufficient. However, many tumours which are apparently localised are, in fact, microscopically disseminated, and unless some detectable chemical substance is released from the tumour and thereby mark its presence, the process of disease may not be diagnosed until recurrence becomes obvious. The natural history of relapses after both surgical or radiotherapeutic approaches has indicated certain groups in whom metastatic disease can be assumed to have occurred at the time of diagnosis in a large percentage of cases.



1. Cis-Platinum, Daunomycin, 5-Fluorouracil, Hydroxyurea, Methotrexate, Vinblastine.
2. Actinomycin D, Cycloheximide, Methotrexate, Nitrogen Mustard.
3. Adriamycin, Asparaginase, Cytosine Arabinoside, Daunomycin, 5-Fluorouracil, Hydroxyurea, Methotrexate, Vinblastine, Vincristine.
4. Adriamycin.
5. Bleomycin, Cis-Platinum, 5-Fluorouracil, Nitrosoureas.
6. ICRF 159
7. Actinomycin D, Adriamycin, Bleomycin, 5-Fluorouracil, Nitrogen Mustard, Vinblastine, Vincristine.

Figure 3 The cell cycle specificity of commonly used cytotoxic agents.

These include breast cancer with positive axillary lymph nodes, large bowel cancers with local infiltration and/or involving the regional lymph nodes, gastric, pancreatic and lung cancers. By its very nature, metastasising disease, even in microscopic amounts, is no longer cureable by surgery or radiotherapy.

Apparent remission of disease, therefore, does not mean that treatment can be stopped at this time, since tumour-cell numbers below 10^9 (equivalent to 1 cm. in diameter) are extremely difficult to detect clinically. Also tumour marker substances can only detect in the order of 10^5 cells and then only in a limited group of conditions. Adjuvant therapy has, therefore, enabled considerable improvement in prognosis in a number of disease states in which the cure rate with surgery and/or radiotherapy was poor, e.g., Wilms' tumour (Farber, 1966; D'Angio, 1972; D'Angio et al., 1973;) Ewing's tumour (Huster et al., 1972; Rosen, 1974;) embryonal rhadomyosarcoma (Donaldson et al., 1973; Ghavini et al., 1975; Pratt, 1972;) osteogenic sarcoma (Jaffe et al., 1974; Rosen et al., 1975; Suton, 1974).

The choice of drug combinations has until recently been made on an empirical basis, as have the critical variables of sequence, schedule and ratio. Extensive use of cell-cycle information in the design of drug combinations has now been made. Combination therapy is now based on a number of general principles:-

a) All the drugs used should have single-agent activity against the tumour.

b) The drugs should have different types of toxicity so that each one can be used in optimal dosage.

c) The combination should be synergistic or at least addictive.

d) The drugs should have different biochemical sites of action and should take effect at different stages of the cell cycle.

e) Agents may also be chosen for their ability to reach certain sites.

Effective combinations of drugs may be obtained by attempting to exploit the principles of 'synchronisation' and recruitment.

The purpose of synchronisation is to gather cells in a certain portion of the cell cycle and then destroy them by an agent or agents which maximally kill at this point. The term recruitment in this instance refers to the situation following chemotherapy when the labelling index, or growth fraction, is found to rise. This increase often being associated with tumour regrowth, i.e., recruitment may be an indirect consequence of cell killing, when the cell depletion leads to a simultaneous recruitment of resting cells back into cycle. By inducing non-dividing tumour cells to proliferate they are being delivered into a more vulnerable phase.

Lankin et al. (1971) have suggested that cell synchronisation techniques could increase therapeutic efficacy in acute lymphocytic leukaemia. They reported a greater therapeutic advantage of a second cycle dependent drug, after partial synchronisation with cytosine arabinoside, than after the second drug alone. Capizzi (1974) has studied a combination of asparaginase and methotrexate, in which he observed a complete remission in nine out of eleven adults with acute lymphocytic leukaemia, all of whom were refractory to prior methotrexate.

Clinical protocols have also been designed along synchronisation lines for solid tumours. Barranco et al., (1973) have observed that continuous infusion of bleomycin for 48 hours produced an increase in labelling index in the nodules of patients with melanoma. Costanzi (1976) attempted to use bleomycin to arrest the cycle at the S/G_2 interphase, and subsequent treatment with methotrexate and hydroxyurea 24 hours later. It had been reasoned that the now synchronised cells would have been released from the biochemical block and transferred back towards the S/G_2 interphase. Activity by the other agents was now predicted to give greater cell kill. Although initially the results were not better than what might have been expected from methotrexate alone, they also reported that by increasing the infusion time and methotrexate dose with citrovorum factor rescue the results have looked more promising. There has been some moderate success in the therapy of testicular cancer using vincristine to produce stathmokinetic arrest followed by bleomycin and adriamycin (Burgess et al., 1975).

The validity of this approach has, however, been questioned, since there is no evidence to suggest that synchronisation does occur to any great extent in vivo. Dethlefsen et al., (1977) have stated that whereas partial synchronisation is real, it is poor and desynchronisation occurs within one cycle traverse. They suggest that to obtain any advantage from this procedure requires that the second drug be administered early when the initial cells are tightly grouped. Van Putten (1975) has pointed out that tumours are likely to contain cells with a range of different cell cycle properties. It is therefore unlikely that a tumour which contains a large population of resting cells will be effectively destroyed by this type of therapy.

The Mechanism of Action of Some Commonly Used Antineoplastic Agents

At present there are a great many drugs employed in the fight against malignant disease. A very broad and brief classification of some commonly used agents is outlined in Table.1 Since these drugs have now established places in chemotherapy, and to contrast their mode of action with that of methotrexate, their mechanism of action will be briefly discussed.

ALKYLATING AGENTS

The vesicant action of sulphur mustard on skin, eyes and respiratory tract gained wide medical attention during World War I. The pertinent observation that sulphur mustard also caused leukopaenia, aplasia of the bone marrow and dissolution of the lymphoid tissue coming soon after. Later, studies of the biological nature of nitrogen mustard indicated considerable promise as an antineoplastic agent. The cytotoxic action on lymphoid tissue, particularly, prompted the initiation of clinical studies which established the usefulness of the alkylating agents (Gilman, 1963).

These compounds react co-valently with many cell constituents, however their main site of attack is probably by inducing intra- and inter-strand linking of DNA which subsequently loses template activity. Alkylating agents tend to be phase-specific in their action on the cell cycle for this reason. The active locus in these agents appears to be a carbonium ion or ions which reacts with great avidity with negatively charged molecules such as nucleic acids. Alkylating agents react with non-dividing cells but their cytotoxic action becomes manifest only once the cells begin to divide.

Cyclophosphamide:

This compound is a cyclic N-phosphorylated derivative of nitrogen mustard. It was first synthesised in an attempt to obtain a compound with greater antitumour specificity (Arnold and Bourseaux, 1958). It was soon shown to be active in experimental and clinical cancer (Arnold et al., 1958; Gross and Lambers, 1958).

Table 1 The mechanism of action of commonly used cytotoxic agents

Class	Agent Type	Drug e.g.	Mechanism
Alkylating Agents	Nitrogen Mustards	Cyclophosphamide	Cross-link DNA
	Nitrosoureas	Carmustine (BCNU) Lomustine (CCNU) Semustine (Me-CCNU)	
Anti-Metabolites	Folic Acid Analogues	Methotrexate	Inhibits purine biosynthesis
	Pyrimidine Analogues	5-Fluoro-uracil (5-FU)	Inhibits dTMP biosynthesis
		Cytosine Arabinoside	Inhibits DNA-polymerase
	Purine Analogues	6-Mercapto-purine (6-MP)	Inhibits purine ring biosynthesis & nucleotide interconversions
Natural Products	Vinca Alkaloids	Vincristine (VCR) Vinblastine (VLB)	Inhibits function of microtubules
	Antibiotics	Actinomycin D Adriamycin	Intercalate with DNA: prevents RNA biosynthesis
		Bleomycin	Damage DNA & prevent repair
	Enzymes	L-Asparaginase	Deaminates asparagine, inhibits protein biosynthesis
Miscellaneous Agents	Substituted Ureas	Hydroxyurea	Inhibits ribonucleotide reductase
	Hydrazines	Procarbazine	Undetermined
Hormones	Adrenocortico-steroids	Prednisone	Lympholytic: suppresses mitosis
	Progestogens	Hydroxy-progesterone caproate	Antagonism of endogenous hormones on dependent tumours
	Oestrogens	Diethyl-stilbestrol	
	Androgens	Fluoxy-mesterone	

Cyclophosphamide is inactive as such and must undergo biotransformation before it can express its cytotoxic activity. Extravasation outside a vein does not therefore produce tissue necrosis as with nitrogen mustard.

The complex activation of this drug with formation of active metabolites has been the subject of intensive study. Connors et al., (1974) have proposed the following scheme in which the hepatic cytochrome P-450 mixed function oxidase system first converts cyclophosphamide to a 4-hydroxycyclophosphamide. This in turn is in equilibrium with its acyclic tautomeric form, aldophosphamide. The 4-hydroxycyclosphosphamide can also be converted by dehydrogenation to yield 4-ketocyclophosphamide. This product can be further converted enzymatically by liver aldehyde oxidase, and other aldehyde metabolising enzymes, to carboxyphosphamide. Aldophosphamide can also be cleaved by aβ-elimination reaction to yield phosphoramide mustard and acrolein, both of which are highly toxic.

The active metabolite adophosphamide has been well characterised (Struck and Hill, 1972) but the other metabolites may contribute to the over-all cytotoxic effect. The 4-hydroxycyclophosphamide has not been shown to be efficacious but may still prove to be so (Takamizawa et al., 1973). Phosphoramide mustard has, however, been shown to have antitumour activity against tumours and has also shown some activity in clinical trials (Nathanson et al., 1967). As expected, cyclophosphamide mediates an effect in the G_2 phase of the cell cycle (Klein, 1972).

Nitrosoureas:

The first nitrosoureas were synthesised by Johnston and his co-workers in 1963 (Johnston et al., 1963). Three nitrosoureas have come into clinical use; these are:-

1,3-bis 2-chloroethyl-1-nitrosourea (Carmustine, BCNU),

1,2-chloroethyl-3-cyclohexyl-1-nitrosourea (Lomustine, CCNU),

1,2-chloroethyl-3-4-methylcyclohexyl-1-nitrosourea (Semustine, methyl - CCNU).

These compounds have the unique property of crossing the blood brain barrier (Oliverio, 1973).

Nitrosoureas can react with biologic macromolecules in several ways. The chloroethyl portion of the molecule has alkylating ability and BCNU having two such groups, is capable of crosslinking DNA (Wheeler and Chumley, 1967). Other modes of action include carbomoylation by the isocyanate portion of the molecule and inhibition of DNA repair by the isocyanate metabolite (Kann et al., 1974). As with cyclophosphamide, nitrosoureas delay progression or arrest cells in the G_2 phase of the cell.

ANTIMETABOLITES

Folic Acid Analogues : Methotrexate (MTX):

The mechanism of action of MTX will be discussed in some detail later in this chapter.

Pyrimidine Analogues : 5-Fluorouracil (5-FU):

This was originally synthesised by Duschinsky et al., (1957) and was shown to be a potent inhibitor of thymidylate synthesis. However, its cytotoxic action is not limited to the phase of DNA synthesis (S-phase) and it is active throughout the cell cycle. Activation of 5-FU proceeds to the formation of 5-fluorouridine monophosphate and 5-fluorodeoxyuridylate (Kent and Heidelberger, 1972). In the presence of the co-factor N-5, 10- methylene tetrahydrofolate, 5-fluorodeoxyuridylate binds strongly to the enzyme's active centre. In fact the binding is quite stable and appears to be covalent in nature (Santi et al., 1974). Although considered to be of secondary importance, 5-fluorouridine monophosphate is readily incorporated into RNA, altering RNA synthesis and function, and ribosomal stability.

Cytosine Arabinoside:

The synthetic pyrimidine nucleosides of which cytosine arabinoside is a member, are a class of nucleosides having D-arabinose as the pentose moiety. The major biochemical mechanism leading to cytotoxicity has been ascribed to competitive inhibition of DNA polymerase (Monparler, 1972). Arabinoside cytidine triphosphate is also incorporated into DNA leading to premature termination of the nucleic acid chain (Monparler, 1969). Creasey (1975) has confirmed that incorporation into DNA and RNA occurs in human leukaemic cells. Cytosine arabinoside has been shown to delay progression from the S-phase to G_2 -phase in the cell cycle (Karon and Shirakawa, 1970).

Purine Analogues : 6-Mercaptopurine (6-MP)

A large number of natural purine base analogues have now been prepared and studied. The first clinically effective antipurine was 6-MP which was synthesised by Elion et al., (1959) and was found to have significant activity against human leukaemias (Burchenal et al., 1953).

A variety of biochemical effects of this agent have been found in both normal and malignant tissues. It has been shown that 6-MP is enzymically converted into di- and tri-phosphates (Way et al., 1959) and can be incorporated into both DNA and RNA although the incorporation is small. It also inhibits the incorporation of formate (Skipper, 1954; Heidelberger, et al., $1960_{(a)}$) and glycine (Le Page and Greenless, 1955). As expected 6-MP shows activity in the G_1 phase of the cell cycle (Vandevoorde, et al., 1970).

NATURAL PRODUCTS

Vinca Alkaloids:

Nobel et al., (1958) working on extracts of the periwinkle were unable to confirm the alleged hypoglycaemic activity. They did, however, observe granulocytopaenia and bone-marrow suppression in their rats. Fractionation of such extracts have subsequently yielded four active alkaloids of which vinblastine (VLB) and vincristine (VCR) have received extensive clinical investigation. Both VLB and VCR bind to mirotubules (Benesch and Malawista, 1969) and cause dissolution of the mitotic spindle apparatus (Sartorelli and Creasey, 1969). As expected, these drugs most acutely influence the cell when in metaphase (Palmer et al., 1960; Cardinali and Mehrota, 1963).

Antibiotics : Actinomycin D:

The first crystalline antibiotic agent to be isolated from a culture broth of streptomyces was actinomycin-A (Waksman and Woodruff, 1940). Since then many related compounds have been isolated including Actinomycin-D. The actinomycins are chromo-peptides, most of them containing the chromophore actinocin.

The cytotoxicity is due to its ability to bind to DNA and inhibit RNA synthesis. These complexes require the presence of deoxyguanosine and apparently intercalation of the antibiotic occurs between a base-paired dG-dC sequence. Alterations in the physical properties of DNA are induced by these complexes (Reich, 1963). Evidence has been presented which suggests that actinomycin D acts at the G_2 phase of the cell cycle (Klein, 1972) whereas earlier workers have indicated activity at the G_1 and G_1/S boundary (Kishimoto and Liberman, 1964) and at the S/G_2 boundary (Epifanova et al., 1969).

Adriamycin:

Adriamycin (ADR) is a glycosidic anthracyclene antibiotic and is a fermentation product of Streptomyces pencetius var-caesius. It intercalates between base pairs of DNA, the amino sugar daunomycin playing an essential role in this binding. The DNA helix is twisted to prevent intercalation, producing a larger, thinner molecule and causing inhibition of template activity (Di Marco et al., 1969; Pigram et al., 1972). Adriamycin has been shown to be active at the S-phase (Hittelman and Rao, 1974) and at the S/G_2 boundary (Epifanova et al., 1969).

Bleomycin:

The bleomycins form a group of water-soluble, basic glycopeptides produced by Streptomyces verticullus (Umezawa et al., 1966). It seems most likely that their cytotoxic action relates to their ability to cause chain schism and fragmentation of DNA molecules. Suzuki et al., (1968) have shown that these drugs react with DNA causing single-strand breaks. Also Umezawa (1973) has shown evidence that bleomycin B_2 in very low concentrations can bind to DNA, possibly by a chemical reaction involving reactive groups on the bleomycin and DNA to cause "nicking". Studies with synchronised cells have indicated that the bleomycins block the cell cycle, causing accumulation of cells, some severely damaged, at G_2 (Hittleman and Rao, 1974). Other studies have shown that cells in mitosis are sensitive to these antibiotics (Umezawa, $1973_{(a)}$; Umezawa, $1973_{(b)}$.

Enzymes : L-Asparaginase (L- asparagine amidohydrolase E.C. 3.5.1.1).

Guinea pig serum was observed to have antitumour activity in some murine tumours (Kidd, 1953). The factor responsible for the suppression of the malignant cells was later discovered to be L-asparaginase (Broome, 1963). Its effect is dependent on the fact that some tumours lack the endogenous capacity to synthesise asparagine. In these cases depletion of plasma asparagine by asparaginase can result in tumour cell death. L-asparaginase appears to be effective at the G_1 phase of the cell cycle (Klein, 1972).

MISCELLANEOUS AGENTS

Substituted Ureas : Hydroxyurea:

This drug was first synthesised in 1869 by Dresler and Stein, but it was not until about sixty years later that the first indications of cytotoxic activity were noted. It was found to produce leukopaenia, anaemia and megaloblastic changes in bone marrow of rabbits (Rosenthal et al., 1928). Hydroxyurea inhibits the enzyme ribonucleoside diphosphate reductase which mediates the conversion of ribonucleotides to deoxyribonucleotides, a probable rate limiting step in the synthesis of DNA. The action of these compounds is therefore specific for the S-phase of the cell cycle (Yarbo, 1968; Krakoff, 1975).

Hydrazines : Procarbazine:

The N-methylhydrazines were originally synthesised as potential monoamine oxidase inhibitors. Antineoplastic effects could, however, be demonstrated in a number of these compounds including procarbazine (Bollag, 1963). The mechanism of action has not, as yet, been determined with certainty although it is known to be metabolised to active compounds, (Weinkam and Shiba, 1978).

HORMONES

That tumours deriving from certain tissues are hormone-dependent has been known since the end of the last century. Following the observation that removal of endogenous production of hormones could induce remissions in patients with certain disseminated cancers (Huggins and Hodges, 1941) there has been extensive study of exogenous hormonal therapy in selected tumours.

Adrenocorticosteroids:

Compounds such as prednisone have been extensively used in treating acute lymphoblastic leukaemia and malignant lymphomas because of their lympholytic effects and ability to suppress mitosis in lymphocytes. Effects of prednisone have been observed in the G_1/S boundary (Ernst and Killman, 1970).

Progestogens : Hydroxyprogesterone caproate:

The main role of this drug is in the chemotherapy of endometrical carcinoma (Kelly and Baker, 1961). These compounds were initially examined because of the concept that carcinoma of the endometrium results from prolonged, unopposed overstimulation of oestrogen (Hertig and Sommers, 1949).

Oestrogens : Diethylstilbestrol:

The predominant use of oestrogens such as diethylstilbestrol is in the treatment of breast carcinoma in post-menopausal women and in metastatic prostratic cancer. McGuire et al. (1975) has shown that these tumours which have detectable oestrogen receptors are more likely to respond to hormonal manipulation than those with no detectable oestrogen receptors.

Androgens : Fluoxymesterone:

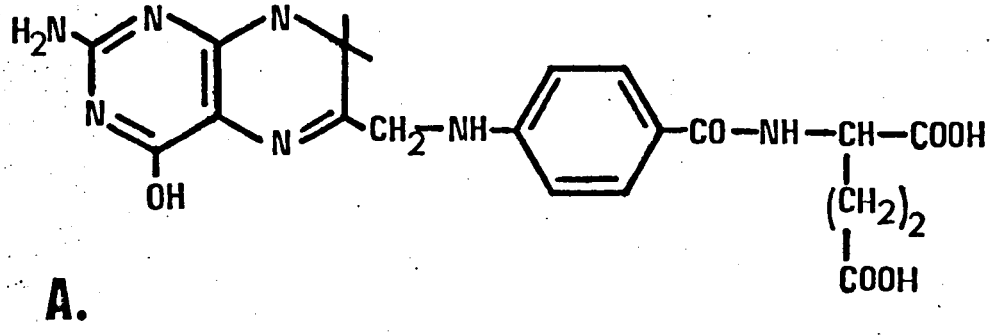
Androgens are used as palliative agents predominantly in pre-menopausal women who have responded to oopherectomy, but they are also useful beyond the menopause.

SECTION 2 : METHOTREXATE PHARMACOLOGY

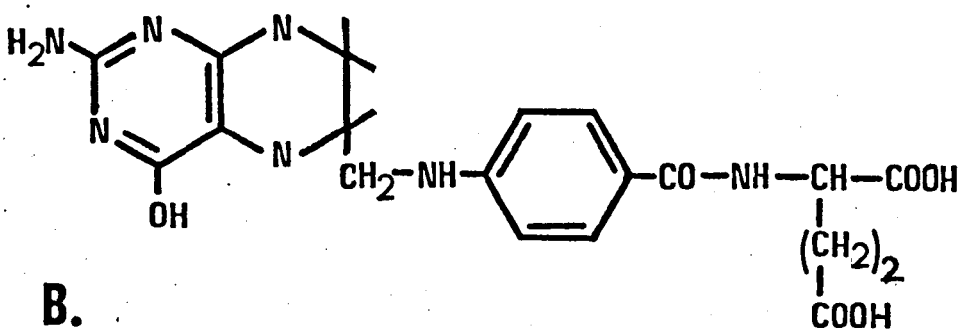
The Mechanism of Action of Methotrexate

Methotrexate (MTX) is an antimetabolite in that by virtue of chemical similarity interferes with the utilisation of a natural metabolite (Fig.4). It inhibits the enzyme dihydrofolate reductase (5,6,7,8 - tetrahydrofolate; $NADP^+$ oxidoreductase. EC 1.5.1.3.) thereby blocking the conversion of dihydrofolate to tetrahydrafolate (FH_4) (Venditti et al., $1960_{(a)}$; Venditti el al., $1960_{(b)}$; Venditti et al., $1960_{(c)}$; Bertino, 1963). The folic acid vitamins serve as growth factors by controlling the metabolism of one carbon compounds, formate and formaldehyde. These are implicit in the biosynthesis of purines, pyrimidines and certain amino acids, transformations generally occurring via co-enzymes derived from tetrahydrofolic acid FH_4 (Fig.5).

The inhibition of dihydrofolate reductase has been described by Werkheiser (1961) as stoichometric, i.e. the inhibitor is so firmly bound to the enzyme that, at levels inadequate to cause complete inhibition, by far the greater portion of the drug is enzyme bound. Also removal of the drug from the enzyme by dialysis against folic acid resulted in precisely equivalent reactivation of the enzyme. While the binding of methotrexate to the enzyme has been shown to be of a reversible and competitive type (Bertino, 1974), the affinity of a partially purified dihydrofolate reductase for methotrexate was found to be about 100,000 times greater than for folic acid (Werkheiser, 1961). Indeed, in earlier work Werkheiser (1959) observed that some 80% of methotrexate activity remained in the supernatant after sucrose fractionation of treated rat liver, which could not be removed by prolonged dialysis. It now also seems likely that methotrexate can persist in tissues, in vivo, for long periods of time without undergoing biotransformation (Fountain et al., 1953). Schrecker et al., (1960) suggested that the sustained antimetabolite activity could be connected to the affinity of drug-enzyme binding. They observed that although incorporation into adenine in leukaemic mice was inhibited over 20 minutes equally by both methotrexate and its dichloroderivative, the action of the former was sustained for longer than the less strongly bound derivative.



A.



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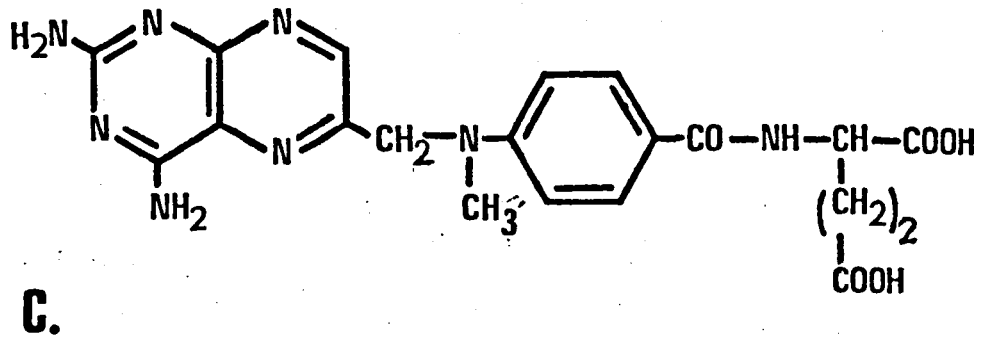


C.

Figure 4 The structures of (A) Dihydrofolic acid (D.H.F.A.)
(B) Tetrahydrofolic acid (T.H.F.A.)
(C) Methotrexate (MTX)

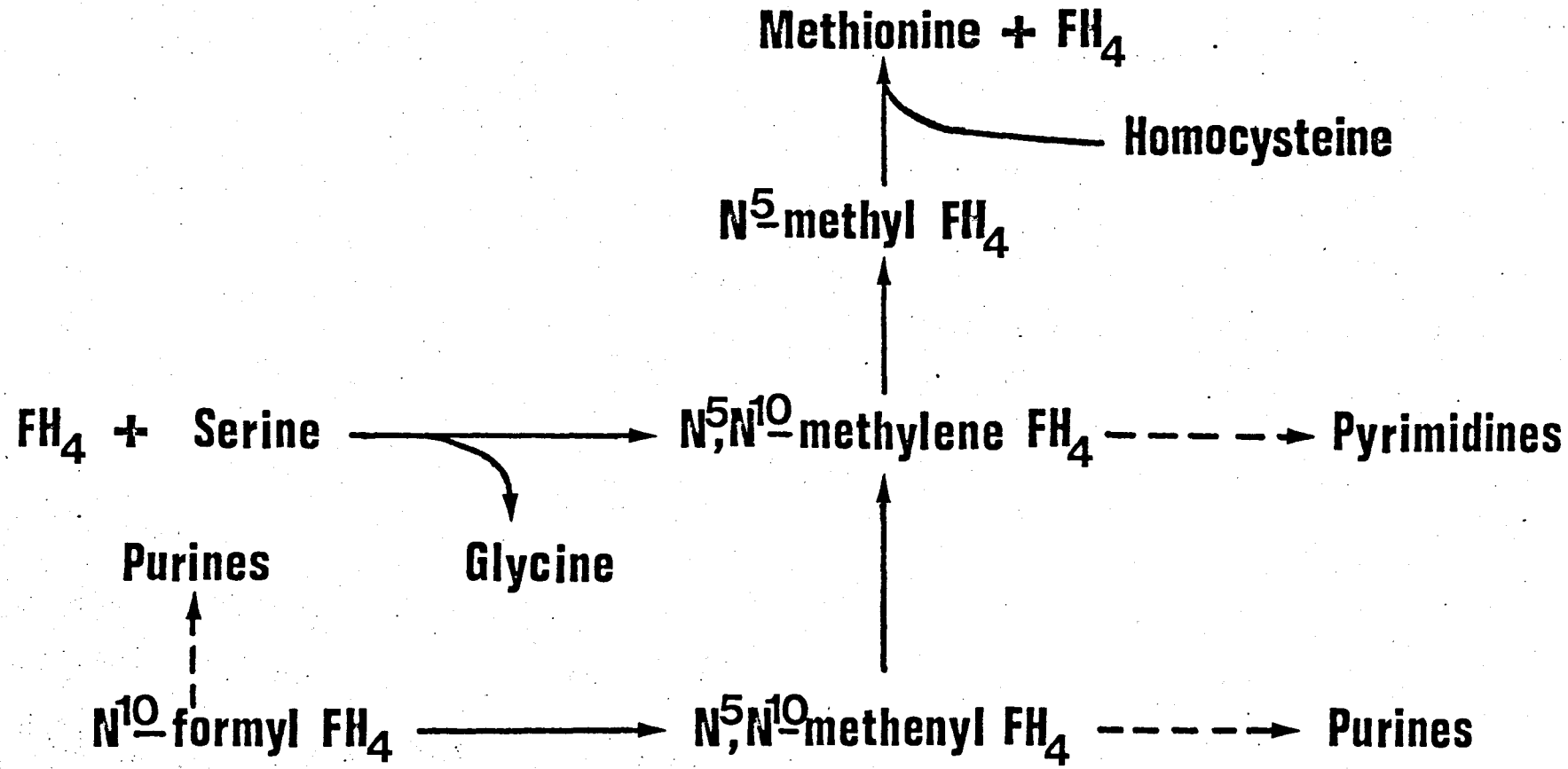


Figure 5 Biochemical transformations dependent on folic acid vitamins

Goldman (1974) has reported observations in L-cell fibroblasts that saturation of high-affinity intracellular binding sites only depressed the incorporation of deoxyuridine into DNA by 27%. Further exposure of cells to methotrexate was necessary to increase inhibition of incorporation. There would appear to be a need to maintain levels above that necessary to saturate the enzyme for maximum effect. Rosenblatt et al., (1978) have recently reported evidence of accumulation of methotrexate polyglutamates by cultured human cells which was associated with prolonged inhibition of DNA synthesis. They have demonstrated that polyglutamate synthesis is dependent on the presence of methotrexate in amounts greater than was necessary for inhibition of dihydrofolate reductase.

Hoffbrand and Tripp (1972) have established, in normal human phytohaemaglutinin - transformed lymphocytes, that the most sensitive locus to depletion of folate coenzymes, due to methotrexate, is thymidylate biosynthesis (Fig.6). This conclusion was based on the observed imbalanced nucleic acid synthesis such that DNA biosynthesis was inhibited to a much greater extent than that of RNA. On the basis of this mechanism methotrexate would be expected to be cell cycle specific, acting predominantly in the S-phase. There is considerable evidence that methotrexate can indeed delay the passage from G_1 - to S-phase and arrest the cycle in S-phase (Rueckert and Mueller, 1960; Ernst and Killman, 1971; Tobey and Crissman, 1972; Mauer, 1975). Ernst and Killman (1971) have reported that doses 1 mg. m^{-2} could arrest leukaemic myeloblasts in the S-phase for about 20 hours but had no direct influence on cells in G_1, G_2 or M-phase. Mauer (1975) has shown that higher doses (30 mg. m^{-2}) can arrest human myeloblasts in the S-phase for a much longer period, exceeding 48 hours, and also slow entry from G_1 into S-phase.

Folic acid coenzymes are also required for the biosynthesis of certain amino acids. The interconversion of glycine and serine has been extensively studied and the involvement of tetrahydrofolate is well established (Deodhar and Sakami, 1953; Blakley, 1954; Alexander and Greenberg, 1955; Jaenicke, 1955).

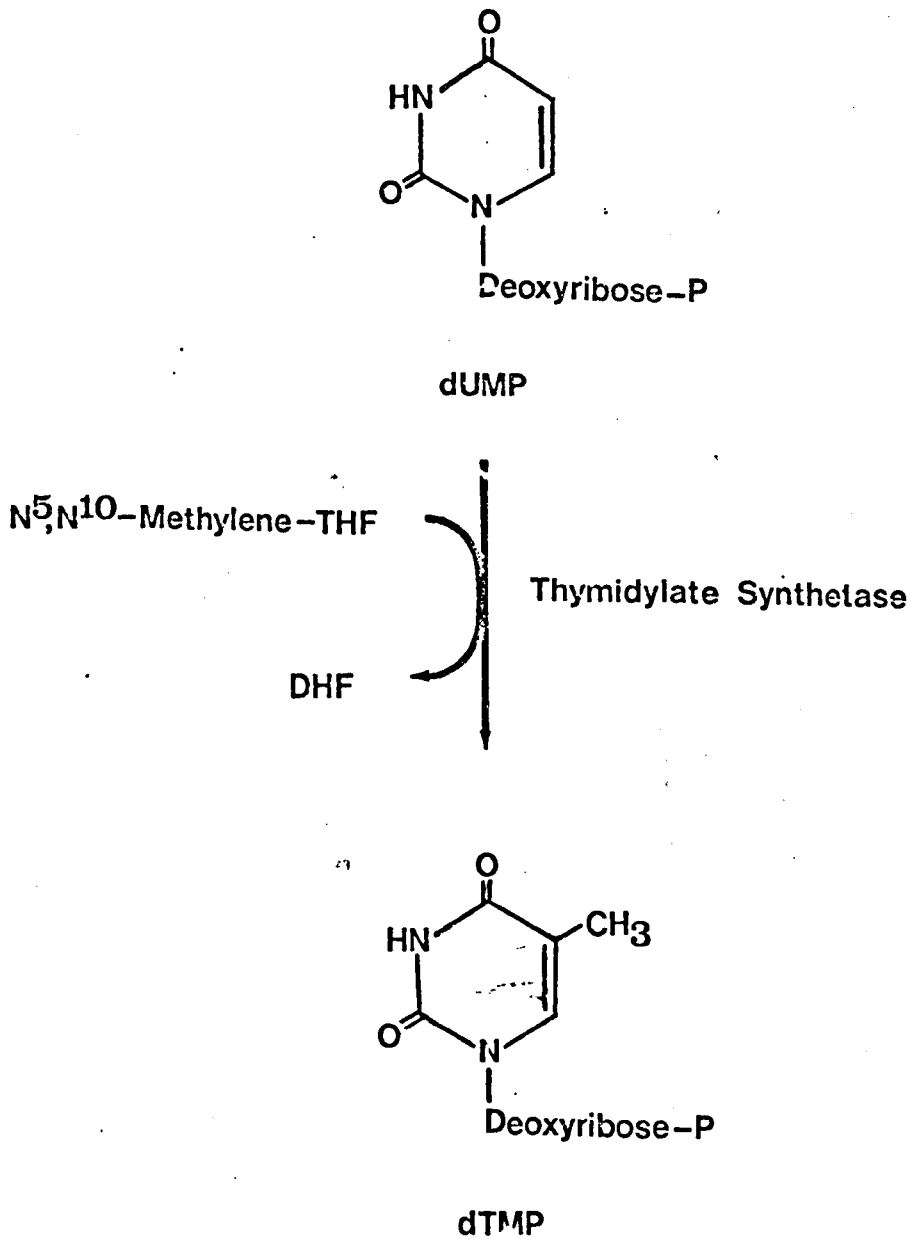


Figure 6 Thymidylate biosynthesis, the most sensitive locus to depletion of folate coenzymes.

Similarly the involvement of tetrahydrofolate in the biosynthesis of methionine from homocysteine has been demonstrated (Nakao and Greenberg, 1955). Bleyer (1977) has pointed out that by preventing amino-acid interconversions methotrexate may also inhibit protein synthesisand may be the mechanism by which high-dose methotrexate can arrest cells in the later stages of the S-phase. There is also some evidence to suggest that reduced folates are necessary for the N- and O-methylation of biogenic amines (Banerjee and Snyder, 1973; Lauduron, 1972). The effect of methotrexate on these systems has not, however, been assessed.

Methotrexate has been demonstrated to mediate an effect at other metabolic sites. In human lymphoblastic cultured cells of the L1210 line these other sites of action include inhibition of thymidylate synthetase and serine hydroxymethyltransferase (Niethammer and Jackson, 1975). Jackson and Niethammer (1977) have, however, dismissed the inhibition of serine hydroxmethyl-transferase as not being significant, since their results indicate the inhibition is too weak. Further, they suggest that the inhibition of thymidylate synthetase would only be effective in the highly drug resistant cells with greatly elevated dihydro-folate reductase where free intracellular methotrexate can accumulate to sufficient levels to exert significant inhibition.

There have also been a number of reports on the effect of methotrexate on the glycolytic enzymes and the metabolic energy state of cells. It has been known since the reports of Warburg (1930), that glycolysis is notably enhanced in cancer cells. Desoize et al., (1978) have studied the induced alteration of glycolysis in L1210 cells in vivo with non-lethal doses of methotrexate. They found that glucose uptake increased and a significant enhancement of key glycolytic enzymes (pyruvate kinase, fructose - 1,6- diphosphate aldolase). Also an increase in ATP concentration was observed to the extent of 2.5 times control values. The results of these workers would suggest a specific induction of key enzymes of glycolysis, but they offer no explanation why glycolysis is not inhibited by the intracellular ATP accumulation.

Kaminskas and Nussey (1978) used Ehrlich ascites carcinoma cells in their tissue culture studies, which are known to be dependent on glycolysis for generation of metabolic energy in the form of ATP. At growth inhibiting concentrations of methotrexate they observed an inhibition of glycolysis and a decrease in adenylate pools which could be overcome by either hypoxanthine or thymidine. Since the effect on cell viability were also found to be somewhat dependent on the concentration of glucose, they concluded that a major cytotoxic effect of MTX appeared to be due to an energy-depleted state. They further suggested that the potentiation of MTX effect by hypoglycaemia and the antagonism of hyperglycaemia may also have therapeutic implications.

Inhibition of transport of reduced folates by methotrexate may also play a positive role in its cytoxicity. In leukaemia L1210 cells the same transport mechanism has been reported to be responsible for the uptake of 5- methyltetrahydrofolate (5- Me FH4), citrovorum factor (C.F.) and MTX (Nahas et al., 1972; Rader et al., 1974). Since these compounds compete with each other, the effect of MTX in limiting the uptake of these reduced folates should augment the principal action on dihydrofolate reductase. By virtue of the similar transport specificities it might be feasible to exploit carrier mediated transport mechanisms to induce selective tumour deficiencies in folate co-factors. Nahas and Bertino (1970) have already shown that 2,4 - diamino derivatives with a methyl group on N-2- position, are poor inhibitors of dihydrofolate reductase, because of steric interference, but are good inhibitors of transport.

Metabolism of Methotrexate

A partially purified enzyme from rabbit liver which catalysed the 7-hydroxylation of MTX was originally described by Johns et al., (1964). They referred to this enzyme as methotrexate oxidase but it was later shown to be identical to aldehyde oxidase (Johns et al., 1965) which was first described by Knox (1946) and later by Rajagopalan (1962). Johns and Loo (1967) confirmed that oxidation by this enzyme occurred only on the 7-position to form the 7-hydroxymethotrexate (7-OHMTX) metabolite (Fig. 7).

This metabolite remained undetected at conventional dose levels and led to the erronious conclusion that methotrexate was not metabolised to any significant extent in man, and was excreted un-changed (Freeman, 1958; Johns et al., 1964; Henderson et al., 1965). Recently it has been identified in urine after high doses of MTX (Jacobs et al., 1976). The aldehyde oxidase capable of this bio-transformation has been found in human liver but not in kidney (Jacobs et al., 1976). These workers have also quantified the amount of metabolite in urine, and have shown that in early time intervals after intravenous MTX (5-200 mg. Kg^{-1} infusion over 6h) the excretion of metabolite constitutes from 0.7 - 9.6% of total drug excreted, whereas at later times (18 - 24h), it can be as much as 7 - 30%. Johns and Loo (1967) have shown, using a partially purified preparation of dihydrofolate reductase from L1210 mouse leukaemia cells (Bertino et al., 1964), that 7-OH MTX is two orders of magnitude less effective as an inhibitor than is MTX.

It has also been proposed that MTX is metabolised by intestinal bacteria. Zahaska et al., (1969), reported that significant amounts of MTX metabolites are present in both urine and faeces of mice and rats after intraperitoneal administrations. Since the amounts of these metabolites were significantly reduced in germ-free mice and in mice pretreated with neomycin, it was considered likely that intestinal bacteria were responsible for the biotransformation. Valerino et al., (1972), have made an intensive study of the metabolism of MTX by intestinal flora.

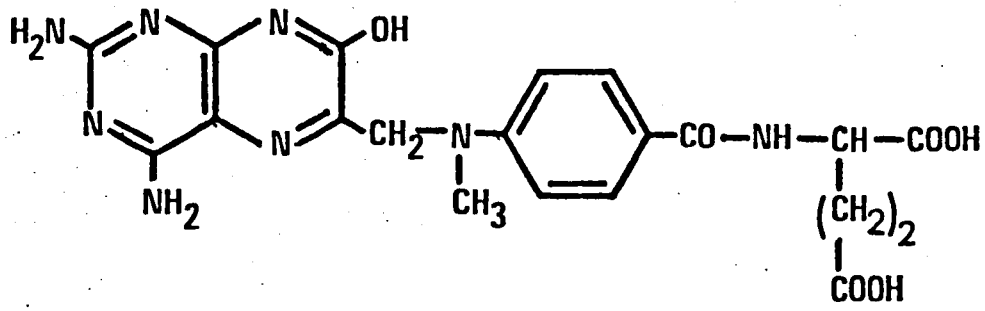


Figure 7 The structure of 7-Hydroxymethotrexate (7-OHMTX)

By incubating MTX with caecal contents of mice they were able to demonstrate at least three metabolites. The principal metabolite was identified as 4-amino - 4-deoxy - N,10 - methylpteroic acid (APA), and it could be recovered from urine and faeces of mice after intraperitoneal injection. The metabolite was determined to be a moderate inhibitor of dihydrofolate reductase with a similar affinity to 7-OH-MTX, but was considerably less toxic than MTX in mice. It could also act as a substrate for aldehyde oxidase yielding 4-amino - 4-deoxy - 7-hydroxy - N,10 - methylpteroic acid (7-OH-APA). A metabolite has been observed, in small amounts, in human urine with elution characteristics on DEAE cellulose similar to this pteroic derivative (Johns and Valerino, 1971; Huffman et al., 1973). A number of workers have reported that metabolites account for less than 10% of the total administered dose if the drug is given intravenously at 30 mg. m^{-2} (Anderson et al., 1970; Huffman et al., 1973; Wan et al., 1974). However, if this same dose was given orally as much as 35% may be excreted as metabolites (Wan et al., 1974).

Polyglutamate derivatives have also been found in human liver (Jacobs et al., 1975). Rosenblatt et al., (1978), have examined the accumulation of MTX polyglutamates in cultured human fibroblasts and obtained evidence that the metabolite may be responsible, at least in part, for prolonged inhibition of DNA synthesis.

Biochemical Resistance to Methotrexate

The term 'resistance' describes a variety of biochemical and pharmacological phenomena. 'Natural resistance' may be defined as lack of tumour response to a given drug regimen despite significant host toxicity, and is often referred to simply as insensitivity. The emergence during or after treatment of a cell population that is less sensitive to the drug than was the original population is known as 'acquired resistance'. Although the therapeutic history of the cells involved is different in cases of natural resistance and acquired resistance, the types of biochemical mechanisms responsible for this failure to respond to drug treatment are fundamentally the same.

Resistance to MTX therapy has been attributed to one or more of three mechanisms:-

1) Decreased ability to transport the drug across the cell membrane.
2) Synthesis of more dihydrofolate reductase and increased cellular activity.
3) Synthesis of an aberrant dihydrofolate reductase insensitive to methotrexate due to a change in enzyme affinity for the drug.

There is extensive evidence in the literature that both natural and acquired resistance may be due to failure of the target cells to accumulate sufficient MTX. Most of the model systems have employed leukaemic cells invitro and attempted comparison with response in vivo (Kessel et al., 1965; Kessel et al., 1968). Ryser and Shen (1978) appraised the use of MTX conjugated to poly-(L-Lysine) in overcoming transport difficulties and therefore drug resistance in culture Chinese hamster ovary cells. The conjugate appeared to be taken up by pinocytosis (Shen and Ryser, 1978; Ryser et al., 1978) and although the conjugate itself is not active in inhibiting dihydrofolate reductase, it has been shown by these workers to be degraded within the cell releasing native methotrexate. They have also shown that absorption by this method is comparable to unconjugated MTX in cells with proficient transport capability.

Several reports on the nature of the increase in enzyme concentration and the mechanism of the increase in activity have appeared. Moderately resistant mutants of L1210 mouse leukaemia cells showing a seven-fold increase in dihydrofolate reductase activity were also shown to synthesise the enzyme at seven times the normal rate, but to degrade the enzyme at the normal rate (Jackson and Huennekens, (1973); Huennekens et al., 1973; Jackson et al., 1975). A highly resistant L1210 mutant has also been observed with an enzyme level eighty times higher than expected, which not only synthesised the enzyme at a greater rate but also degraded it slower than normal (Jackson and Huennekas, 1973). Further characterisation of this enzyme showed that it had different electrophoretic properties from normal L1210 enzyme and it bound NADPH with greater affinity (Jackson et al., 1973; Niethammer et al., 1973). An increase in total dihydrofolate reductase with altered properties has also been reported in a mouse leukaemia L4946 subline (Blumenthal and Greenberg, 1970).

Some studies have indicated that varying intrinsic sensitivity to MTX of different mammalian cell types may correlate with the affinity of the binding of MTX by the cell's dihydrofolate reductase (Harrap et al., 1971; Jackson et al., 1976). Clear differences in the natural resistance between Yoshida sarcoma and L1210 leukaemia cells have been demonstrated, which correlated with the affinity of the enzyme drug binding (Jackson, 1976). Despite the fact that the two cell lines had similar activities of dihydrofolate reductase and similar rates of membrane transport of MTX, the Yoshida sarcoma cells were seventy times more resistant than the leukaemia cells and the enzyme from the L1210 leukaemia cells bound MTX twenty-five times more tightly.

Jackson and Niethammer (1977) have also described the phenomenon of acquired resistance in human lymphoblastoid cells resulting from altered kinetic properties of the reductase. They observed that the affinity for MTX was reduced by fifty-fold, which at low MTX concentrations would provide these cells with a selective advantage. However, they point out that at high intracellular concentrations of MTX these cells would be compromised, since it would be expected that free MTX could accumulate to sufficient levels to exert significant inhibition of thymidylate synthetase.

Methotrexate Toxicity

Methotrexate chemotherapy is limited by the overt toxicity which it can promote. The most commonly encountered toxicities are myelosuppression; gastrointestinal mucositis and hepatitis. Other toxic reactions generally depend on the regimen imposed on the patient. These are broadly outlined in Table.2

Nephrotoxicity which has been observed with high dose therapy (Condit et al., 1969) is particularly vexing since MTX can be retained due to the renal dysfunction and further toxicity may ensue (Frei III, 1976).

Chemical arachnoiditis associated with intrathecal MTX is a distressing acute syndrome characterised by headache, backache, vomiting, fever, meningismus and a cerebrospinal-fluid pleiocytosis (Mott et al., 1972). Motor dysfunction of the brain or spinal column is occasionally manifest, and may include paraplegia, quadriplegia, cerebellar dysfunction, cranial nerve palsies and seizures (Gagliano and Costanzi, 1976; Weiss et al, 1974; Weiss and Kahn, 1978). Since these neurological syndromes occur in the presence of elevated C.S.F. concentrations of MTX (Weiss and Kahn, 1978; Bleyer et al., 1973), it has been suggested that frequent monitoring of C.S.F. drug concentrations may be predictive of serious neurotoxicity, and drug dosage and drug interval could be adjusted accordingly (Beyer et al., 1973). However, neither the minimal effective concentration of MTX in C.S.F. nor the toxic concentration is known (Shapiro et al., 1975) and therefore can not serve as a safe guide in treatment or prevention of neurological complications.

A necrotising demyelinating leukoencephalopathy has also been well described (Bresnan et al., 1972; Kay et al., 1972; Shapiro, 1973; Hendin et al., 1974; Norrell et al., 1974; Smith, 1975; Price and Jamieson, 1975) and is reflected in the clinical pattern of progressive neurological deterioration (evolving into severe dementia, dysarthria, ataxia, spasticity, seizures and coma). Price and Jamieson (1975) found a direct correlation between the incidence of leukoencephalopathy and the cumulative dose of MTX.

REGIMEN	REFERENCES
Chronic Low Dose Systemic Administration	
Cirrhosis/focal necrosis	Ryan et al (1972); Tobias and Auerbach (1973); Nesbit et al (1976).
Intestinal pneumonitis	Nesbit et al (1976).
Osteoporosis	Nesbit et al (1976).
Alopecia	Barranco (1972).
Immunosuppression	Barranco (1972); Thomas and Storb (1971).
Pulmonary reaction	Clarysse et al (1969); Everts et al (1973); Lascari et al (1977).
High Dose Systemic Administration	
Renal dysfunction	Condit et al (1969).
Vomiting	Jaffe and Traggis (1975).
Acute desquamous dermatitis	Pitman et al (1975).
Immunosuppression	Mitchell et al (1969).
Reactivation of solar dermatitis	Corder (1976).
Vasculitis of hands and feet	Lanzkowsky (1976).
Conjunctivitis	Steele et al (1979).
Intrathecal Administration	
Chemical arachnoiditis	Mott et al (1972).
Motor dysfunction	Gagliano and Costanzi (1976); Weiss et al (1974); Weiss and Kahn (1978).
Leukoencephalopathy	Bresnan et al (1972); Hendin et al (1974); Kay et al (1972); Norrell et al (1974); Shapiro (1973); Smith (1975).
Seizures	Kay et al (1972); Smith (1975).

Table 2 Common manifestations of methotrexate toxicity.

The maximum tolerated dose of MTX varies widely from person to person, e.g., Hanser et al., (1971), have reported that this can vary from 80-900 mg. m^{-2}. Further, the study of MTX pharmacokinetics has been able to assert the role not only of concentration, but also the dependence of drug exposure time to toxicity. If the concentration/time threshold is exceeded toxicity will ensue. The threshold for a few tissues have been estimated and found to be profoundly different. For bone marrow and gastrointestinal epithelium the thresholds appear to be about 2×10^{-8} M and 42 hours (Levitt et al., 1973; Young and Chabner, 1973), and for lung (Sostman et al., 1976) and liver (Podurgiel et al., 1973) the plasma concentration threshold is similarly relatively low, being 10^{-8} - 10^{-7}M. The concentration threshold for kidney is probably dependent on urine pH, but appears to be quite high at about 10^{-4}M (Stoller et al., 1975). Skin and brain also appear to have a relatively high tolerance to intravenous MTX, with a concentration threshold in the region of 10^{-5}M (Weinstein, 1977). Pulmonary toxicity on the other hand has been obtained after weeks to months of oral therapy and rarely with intermittent high-dose intravenous therapy (Nesbit et al., 1976; Sostman et al., 1976; Wall et al., 1977). The severity of toxicity also seems to be directly proportional to the duration of MTX exposure beyond the time threshold, and less dependent on the magnitude of MTX elevation above extracellular concentration threshold (Goldie et al., 1972; Chabner and Young, 1973).

Over the past few years a number of compounds have been examined in an attempt to increase host tolerance without loss of therapeutic effect of MTX. The use of citrovorum factor (folinic acid; 5-formyltetrahydrofolic acid) (Fig. 8) is now well established and is obligatory in high dose MTX regimens. The first suggestion that therapeutic effectiveness might be increased by administering high doses of MTX with citrovorum factor was made by Goldin et al., (1966) in leukaemic mice. The rational for high dose therapy is to produce serum levels of the drug high enough to permit facilitated diffusion into tumour cells deficient in transport sites, and subsequently permit the accumulation of sufficient MTX to saturate

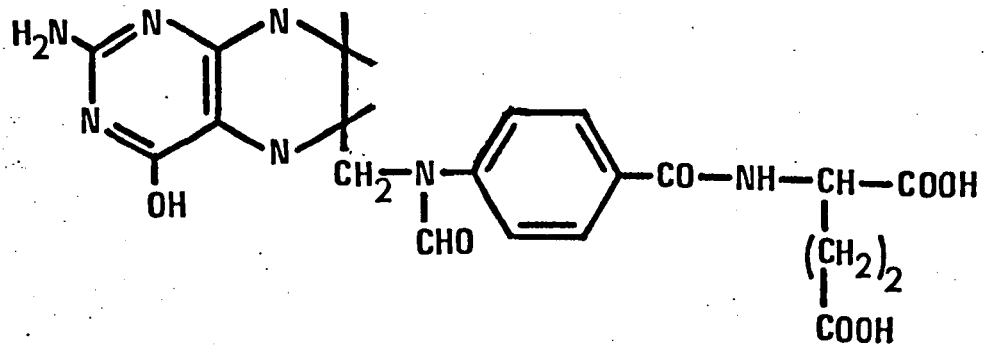


Figure 8 The structure of citrovorum factor
(C.F., folinic acid, 5-formyltetrahydrofolic acid)

all the high affinity sites on dihydrofolate reductase, and ideally enough free MTX to bind to low affinity sites thus hopefully circumventing the excess production of dihydrofolate reductase by the resistant cell. It has been demonstrated that when citrovorum factor is given after MTX it can prevent much of the toxicity without reversing the oncolytic effect of the drug (Levitt, et al., 1973; Bender, 1975(a); Bender, 1975(b)). It has been possible to show that citrovorum factor may preferentially rescue normal cells (Frei et al., 1975) and also that those tumour cells which are resistant to MTX because of a defective transport system are also resistant to citrovorum factor 'rescue' since both drugs share the same transport system (Goldman, 1971; Nixon and Bertino 1972).

Bender (1975) has indicated three basic elements of citrovorum factor rescue:-

1) The interval between the MTX dose and folinic acid administration.
2) The total dose of folinic acid administered.
3) The period over which the dose is administered.

Alteration of these parameters may significantly alter the therapeutic index of the dual drug administration. Pharmacologically citrovorum factor by-passes the MTX induced inhibition of dihydrofolate reductase since it can enter the folate cycle directly without prior reduction by this enzyme(Fig.9). Mead et al., (1963) have shown that tetrahydrofolate and 5-methyltetrahydrofolate are also capable of reversing the toxicity and antileukaemic effect of MTX in mice. Blair and Searle (1970) have also demonstrated that 5-methyltetrahydrofolate can protect against the lethal effects of repeated injections of MTX to mice, and further that this protection did not differ greatly from that obtained using citrovorum factor. This is perhaps not surprising since Blakely (1969) has shown that 5-methyltetrahydrofolic acid is the main storage form of folates in the body. More recently Kishuik et al., (1977), have substantiated that other reduced folates can prevent MTX activity in mice. They have observed that dl, L-5, 10-methylenetetrahydrofolate, l, L-5, 10-methylenetetrahydrofolate, dl - 5-methyltetrahydrofolate and dihydrofolate all show activity. However, they found d, l-5, 10-methylenetetrahydrofolate to be inert.

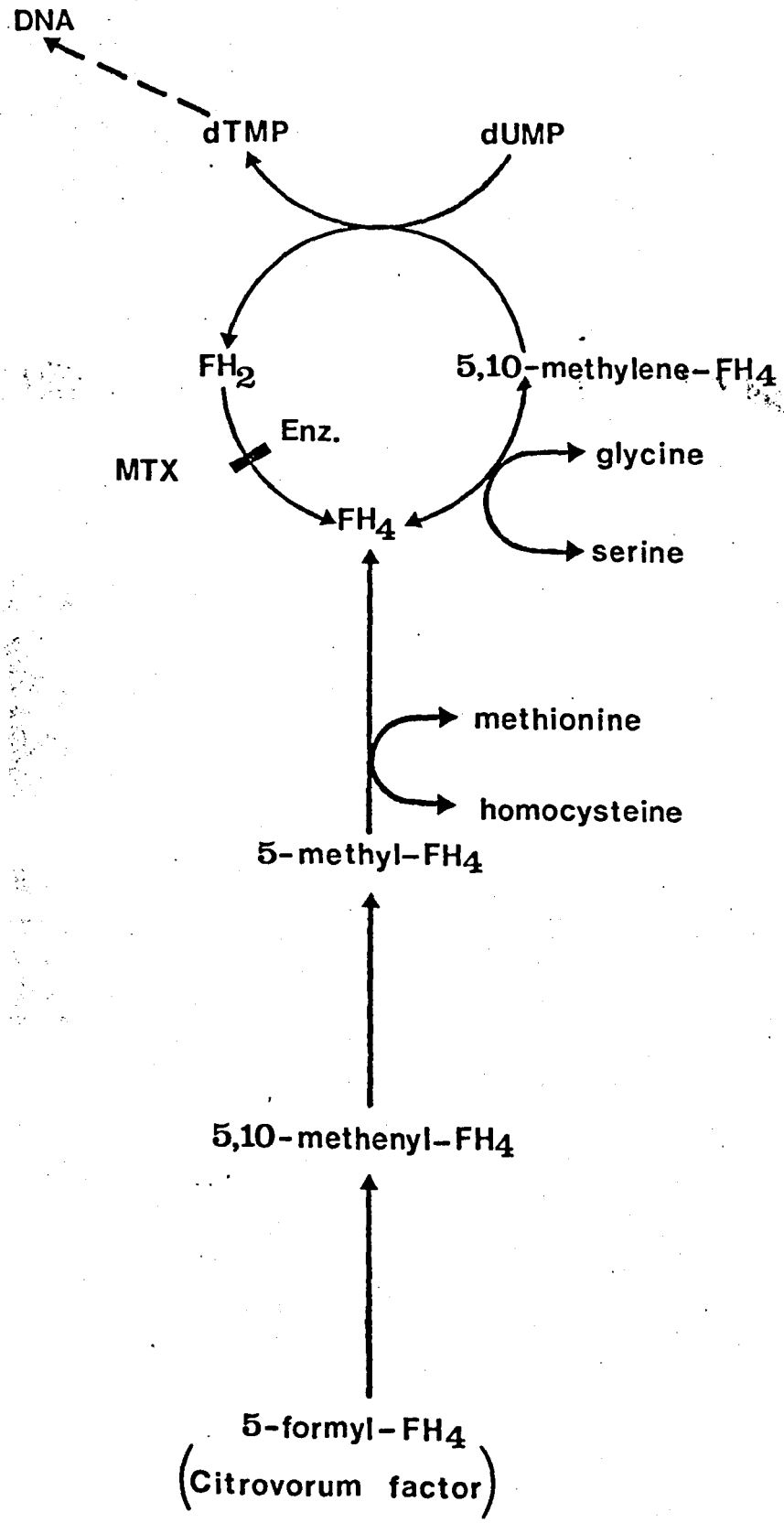


Figure 9 The mechanism of citrovorum factor rescue
Enz: Dihydrofolate reductase

Another potentially important approach to toxicity rescue is not to restore the reduced co-factor pool but to provide the end products of the co-factor dependent reactions. The ability of thymidine to reverse the impairment of DNA synthesis was first demonstrated by Killman (1964) in pernicious anaemia. Whereas citrovorum factor relieves the cell from the three major effects of MTX (inhibition of DNA, RNA and protein synthesis), thymidine selectively rescues cells from inhibition of DNA synthesis. It has been reported that in murine systems, normal marrow and gut cells in vivo can generally be protected by thymidine alone (Seman and Grindley, 1976; Tattersall et al., 1975), whereas some tumours require a source of purines in addition to thymidine (Borsa and Whitmore, 1969; Hryniuk et al., 1975). It is therefore possible that rescue with thymidine may protect normal host tissues while leaving tumour cells partially unprotected which may result in selective cell kill and an improvement in the therapeutic index of MTX. Thymidine has also been shown to have potential in the protection against MTX toxicity in humans (Ensminger and Frei III, 1977; Howell et al., 1978).

If the reduced folate stores in the body are depleted it may therefore influence other methyl transfers, such as those occurring via s-adenosylmethionine which is required for the synthesis of choline. Since choline is the major lipotropic compound in the body, a deficiency could lead to impaired transport of fat from the liver resulting in fatty metamorphosis. Custer et al., (1976) have demonstrated that hepatic lesions comparable to that in man can be induced in rats in which signs of nucleic acid deprivation could also be clearly observed. With this observation as the basis of a model, Tuma et al., (1975) and Freeman-Narrod, (1977) have shown that choline provides protection to the lives of rats after prolonged treatment with MTX. Freeman-Narrod, (1977) also indicated that the therapeutic effects of MTX via inhibition of nucleic acid synthesis did not seem to be impaired by choline rescue.

Further, their results support the above hypothesis that MTX inhibits biosynthesis of choline, at least in rats, leading to fatty metamorphosis and other subsequent lesions in the liver. Methionine has also been shown to provide some protection against hepatoxicity (Yourtree et al., 1977).

It is also possible to rescue patients by lowering the plasma levels of MTX. A method which has been shown to have potential is infusing carboxypeptidase - G_1 (Chabner et al., 1972). This enzyme inactivates methotrexate by molecular cleavage thereby effectively lowering the plasma concentration of available drug.

A BRIEF HISTORY AND CURRENT USAGE OF METHOTREXATE

While the concept of specific drugs active against cancer goes back to the work of Paul Erhlich in the latter part of last century, modern cancer chemotherapy had its beginnings in the 1940's. The observation that oestrogens could induce remissions in some cases of prostate cancer (Huggins and Hodges, 1941) was followed by the use of nitrogen mustard to induce remissions in malignant lymphoma (Gilman and Phillips, 1946). The introduction of folic acid antagonists rapidly followed.

The first antifolates to be synthesised were aminopterin (Seeger et al., 1947) and MTX (Seeger et al., 1949), and although various other analogues with antineoplastic effects have been prepared (Smith et al., 1948) these remain the agents of greatest interest. These folic acid antagonists (methotrexate and aminopterin) have been the subject of extensive laboratory and clinical investigations.

Aminopterin was originally shown to inhibit the growth of Streptococcus faecalis (Seeger et al., 1947; Olesen et al., 1948) and later to retard the growth of transplanted sarcoma in mice (Schoenbach et al., 1949). Great interest was focused on antifolates, particularly after the demonstration, in 1948, that aminopterin was capable of producing temporary remissions in acute leukaemia in children (Farber et al., 1948). Indications that folic acid could reverse the growth inhibitory effects of aminopterin in mice (Franklin et al., 1948), rat and chick (Olsen et al., 1948) was observed, and Goldin et al., (1949) demonstrated that folic acid was also capable of reversing aminopterin-induced inhibition of Sarcoma 180. Folic acid was also found to influence the anti-leukaemic effectiveness of MTX (Burchenal et al., 1949) and some time later folinic acid was shown to have a similar protective effect (Burchenal et al., 1950). Increased attention was directed towards MTX when Goldin et al., (1955) found that it was more effective in the treatment of L1210 leukaemia than was aminopterin.

Methotrexate has since found broad usage in the treatment of a large number of malignancies. Both experimental and clinical studies have shown that both the dose and schedule of administration may have a profound influence on efficacy. Both Goldin et al., (1956) and Skipper, et al., (1957) have suggested that the ability of MTX to totally eradicate a tumour population is related to the dose of drug that can be employed. Although the dose of drug is limited by overt toxicity, the antagonism of such effects with folinic acid has permitted greater manipulation of the basic regimens.

Three basic treatment schedules are currently in use:-

1) Conventional low-dosage without folinic acid
2) High-dosage with folinic acid
3) Intrathecal MTX therapy

Methotrexate was initially administered in doses of 1 to 5 mgm^{-2} until the early 1960's when intermittent parenteral doses of 30 mg. m^{-2} twice weekly was introduced. This regimen was found to be more effective for maintenance therapy of acute childhood leukaemia (Anderson et al., 1970; Selawry and Hansen, 1965). Intermittent low dose therapy has proved to be highly effective against a number of malignant diseases including choriocarcinoma, acute lymphocyte leukaemia and breast carcinoma.

In high-dose regimens with folinic acid rescue, MTX is either administered by infusion over a short time period of 4 to 6 h. or over longer times of 20 to 42 h. (Penta, 1975). In the former infusion regimen an attempt is made to maximise intracellular drug levels in poorly perfused or transport resistant tumours (Anderson et al., 1970).

The rationale behind the longer infusion is to encompass more of the cell-cycle of the neoplastic cells and to reach an equilibrium between plasma concentration and the levels of drug in interstitial and intracellular compartments. Concentrations ranging from 50 mg Kg^{-1} to gram quantities have been employed in high dosage regimens.

Folinic acid rescue is generally initiated between 24 h. and 42 h. after the start of high-dose methotrexate therapy. Significant toxicity may occur if folinic acid 'rescue' is delayed or if discontinued before the plasma MTX level falls below the concentration threshold of vulnerable tissues. The serum MTX concentration is a useful index of when folinic acid 'rescue' can be safely withdrawn (Isacoff et al., 1976; Nirenberg et al., 1976; Stoller et al., 1976). Serum concentrations can also be of use in determining folinic acid rescue dosage (Frei III, 1976; Isacoff et al., 1976; Jacobs and Dihettuso, 1977). Alkalinisation of the urine in patients receiving high doses of MTX is common practise and is useful in preventing nephrotoxicity and without altering myelo-suppression (Patman et al., 1976; Sadoff and Rittmann, 1976).

Such high-dose infusion regimens have been successful in promoting responses in a number of otherwise resistant diseases, e.g, malignant lymphoma (Djerossi and Kim, 1976; Pittman and Frei III 1977) and epidermoid carcinoma of the head and neck (Khandekar and Wolff, 1977; Levitt et al., 1973).

Penetration of MTX into the cerobrospinal fluid (C.S.F.) is poor although objective responses in medulloblastoma and gliomas are obtained from high-dose infusions (Pittman et al., 1975; Kim et al., 1976; Rosen et al., 1977). Intrathecal therapy is an important therapeutic modality recently explored for the treatment of leukaemia and lymphoma (Bagshawe et al., 1969;) the major limitation being neurotoxicity. The conventional dose is 12 mg m^{-2} but a modified dosage schedule has been proposed (Bleyer, 1977). It was observed that C.S.F. levels of MTX in patients receiving the conventional dose are inversely proportional to age. Bleyer (1977) therfore proposed that children under one year of age should receive 6 mg. and that the dose should increase by 2 mg. for each year until three years of age and over, when all patients should be given the same dose of 12 mg.

SECTION 3 : PROTEIN BINDING OF DRUGS

PROTEIN BINDING OF DRUGS

Drugs are carried from their site of injection or absorption to different sites within the body by the circulating blood. Although some drugs are simply dissolved in serum water, e.g. ethanol (Feller and le Petit, 1977), the great majority are associated with blood constituents such as albumin, globulins, and lipoproteins. Bennhold (1938) proposed that serum proteins constituted a specialised transport mechanism essential for the distribution of endogenous substances. Extensive experimentation and examination of the binding of numerous compounds has indicated that serum albumin is the principle component in serum to which drug molecules bind (Goldstein, 1949; Meyer and Guttman, 1968).

Almost every drug mediates its pharmacological influence by interacting with some macromolecule at particular sites in the body, such as specialised receptor sites, transport systems, membranes and enzymes. However, from the standpoint of direct therapeutic effect the sites of action on serum albumin have been termed 'silent receptors'. Nevertheless, it is imperative that drug-protein interactions be studied as anomalies therein could profoundly alter the elimination, distribution and activity of many drugs.

Protein binding is defined as the reversible interaction between a small molecule and a macromolecule. It is analagous to the enzyme-substrate interaction with the fundamental difference that the complex does not decompose to form new products. Although this definition is assumed in virtually all binding studies, there remains the distinct possibility that a small amount of irreversible co-valent association may occur, e.g. Ronwin and Zacchei (1967) have found evidence of some irreversible as well as reversible binding of ethacrynic acid to serum albumin in vitro.

METHODS OF MEASURING BOUND AND UNBOUND DRUGS

Over twenty methods have been employed to study the protein binding of drugs. They can, however, be summarised into two broad categories. Horecker et al (1969) divided them into:-

1) equilibrium methods based upon measuring changes in ligand concentration as a result of the establishment of a binding equilibrium.
2) direct measurement methods in which a property of the drug, protein, or complex may be examined during the binding process.

This classification is based on the principles originally proposed by Goldstein (1949).

Chignell (1971) has categorised them according to:-

1) non-spectroscopic methods.
2) spectroscopic methods.

The first group of both classifications includes techniques such as equilibrium dialysis, ultrafiltration, ultracentrifugation, gel filtration, bioassay, electrophoresis, conductivity and osmotic pressure. The second group includes some of the most complex methods such as, fluorescence spectroscopy, ultraviolet and visible absorption spectroscopy, optical rotatory dispersion, circular dichorism and nuclear magnetic resonance.

Each of these methods has its advantages and limitations which are briefly summarised in Table. 3

	Advantages	Disadvantages
Simple Ulltrafiltration	Thermodinamically sound; rapid; quantitative	Binding to membranes; equilibrium altered by change in protein concentration
Ultracentrifugation	No Donnan inequalities; quantitative	Quantitation can be difficult; alteration of equilibrium due to protein concentration change
Gel Filtration	Thermodinamically sound; separation of binding proteins	Dilution causing alteration of equilibrium due to protein concentration change
Equilibrium Dialysis	Thermodinamically sound; reproducible; quantitative	Donnan inequalities; nonphysiological; possible bacterial contamination and protein denaturation
Bioassay	Historical interest only	
Electrophoresis	Qualitative data; separation of binding proteins	Quantification difficult; binding to support medium
Conductivity	Historical interest only	
Osmotic Pressure	Historical interest only	

Fluorescence Spectroscopy	Qualitative and quantitative; homogenious system	Some drugs may cause no spectral change; minor filter effects; changes not always due to drug/protein equilibrium
Absorption Spectra; Ultraviolet Visible	Sensitive; can detect conformational changes; homogenious system	Some drugs may cause no spectral change; need to compensate for absorbance of compound.
Optical Rotatory Dispersion	Can serve as a probe for binding site; qualitative or quantitative	Interpretation difficult; not always due to changes in association or dissociation of protein drug complex
Circular Dichorism		
Nuclear Magnetic Resonance	Sensitive to changes in molecular geometry	Interpretation difficult

Table 3 The advantages and disadvantages of the most commonly employed methods of studying protein binding.

METHODS OF ANALYSING BINDING DATA

Goldstein (1949) indicated that expressing binding data simply as percentage bound is meaningless unless qualified by an indication of the unbound drug concentration at equilibrium. Even for those drugs where the percentage bound does not vary greatly over the therapeutic range, this method of expressing results is clearly unsatisfactory. A plot of the percentage bound drug against total drug concentration can, however, provide extremely valuable information, since the percentage bound drug decreases with increase in total drug concentration. Therefore, if binding measurements are made at a number of drug concentrations and if the concentration of the binding protein can be determined the following binding parameters can be estimated:

a) The number of classes of binding sites.

b) The number of binding sites in each class, n_1, n_2 etc.,

c) The association constant for each class, K_1, K_2 etc.,

Knowledge of these parameters would characterise the binding between drugs and proteins. It would enable a comparison to be made between the interaction of different drugs and also between data obtained for a particular drug by different methods of measuring bound and free drug. It would be very difficult to interpret a comparison based solely on the value of percentage bound.

The reversible association can be described by the application of the law of Mass Action:-

$$(P) + (Df) \underset{K_2}{\overset{K_1}{\rightleftharpoons}} = (PD)$$

where (P) = free protein concentration

(Df) = free drug concentration

(PD) = drug-protein complex concentration.

K_1 = rate constant for the forward reaction.

K_2 = rate constant for the backward reaction.

At equilibrium, the association constant for this reaction will be:-

$$Ka = \frac{k_1}{k_2} = \frac{(PD)}{(P)(Df)} = \frac{1}{Kd} \text{ (dissociation constant)} \quad \ldots\ldots\ldots 1$$

$$\text{therefore } (PD) = Ka\ (P)\ (Df) \quad \ldots\ldots\ldots 2$$

The quantity 'r' defined as:-

$$r = \frac{\text{moles of drug bound}}{\text{moles of protein}}$$

$$= \frac{(PD)}{(PD) + (P)}$$

substituting Ka (P) (Df) for (PD) from equation 2

$$\text{therefore } r = \frac{Ka\ (P)\ (Df)}{Ka\ (P)(Df) + (P)}$$

$$= \frac{Ka\ (Df)}{1 + Ka\ (Df)} \quad \ldots\ldots\ldots 3$$

This derivation is for one binding site, therefore if there are 'n' independent sites, a series of independent equations can be written and summated:-

$$r + r_2 + r_3 + \text{--------} r_n = r_{total} = \frac{NKa\ (Df)}{1 + Ka\ (Df)} \quad \ldots\ldots\ldots 4$$

Further, there are often more than one type of binding site, i.e. more than one class of binding site, for a particular drug on a given protein, each with its own association constant. This can be expressed in a more general form of equation 3:-

$$r_{total} = \frac{n_1\ K_1\ (Df)}{1 + K_1\ (Df)} + \frac{n_2\ K_2\ (Df)}{1 + K_2\ (Df)} + \frac{ni\ Ki\ (Df)}{1 + Ki\ (Df)} \quad \ldots\ldots 5$$

which has the form of an hyperbola. Since (Df) and (PD) can be measured, r_{total} can be calculated knowing the protein concentration. In this form, therefore, the equation allows for the experimental determination of the binding parameters.

The equation can be rearranged in a number of different ways to facilitate a number of graphical expressions of the data.

As mentioned above, the direct plot of r against (Df) is an hyperbola (Fig. 10). The equation is analogous to the Michaelis - Menten equation. At the plateau r = n, and when $r = \frac{n}{2}$, $Df = \frac{1}{K}$. A satisfactory fitting of experimental data is difficult and therefore accurate values for n and k will not be obtained.

If both sides of equation 4 are inverted and rearranged to give:-

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{nK\ (Df)}$$

a plot of $\frac{1}{r}$ vs $\frac{1}{(Df)}$ should produce a straight line (Fig. 10) analogous to the Lineweaver - Burk plot. In this plot the slope $= \frac{1}{nk}$ and the y intercept $= \frac{1}{n}$. Although this plot can give a good statistical fit it has the disadvantage of heavily weighting data points obtained at low (D) which can lead to misinterpretations of binding behaviour.

A straight line plot of a different type used by Scatchard (1949) is given by the following form of the binding equation:-

$$\frac{r}{(Df)} = nKa - rKa$$

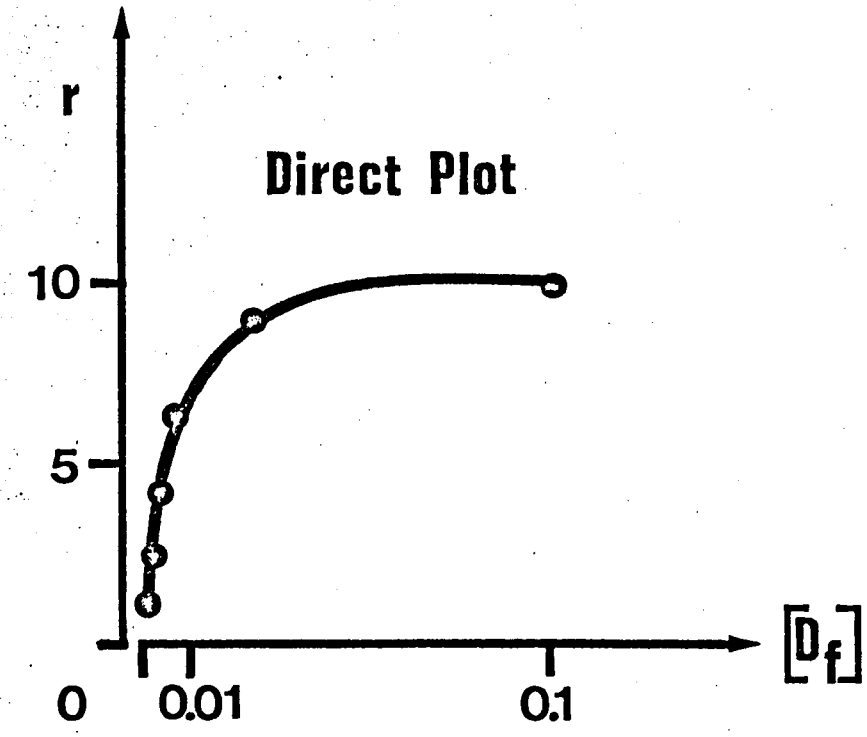
in which $\frac{r}{(Df)}$ is plotted against r. The number if binding sites (n) is determined from the intercept on the abcissa and nK (the product of the association constant and the number of binding sites) from the intercept on the ordinate. This method places less stress on 'r' values at low (Df) giving a more even distribution to the data points. Curvilinear plots are often obtained with this method, (Fig. 11), indicating more than one class of binding sites. These curves can be fitted as the summation of two or more straight lines, each due to a different class of binding sites.

Sandberg and Rosenthal (1967) have discussed the treatment of binding data when the nature and concentration of the protein is not known. The recommended plot is based on the following rearrangement of equation 4:-

$$\frac{(Db)}{(Df)} = n\,K(Pt) - K(Db)$$

where (Pt) = total binding protein concentration

(Db) = concentration of bound drug.



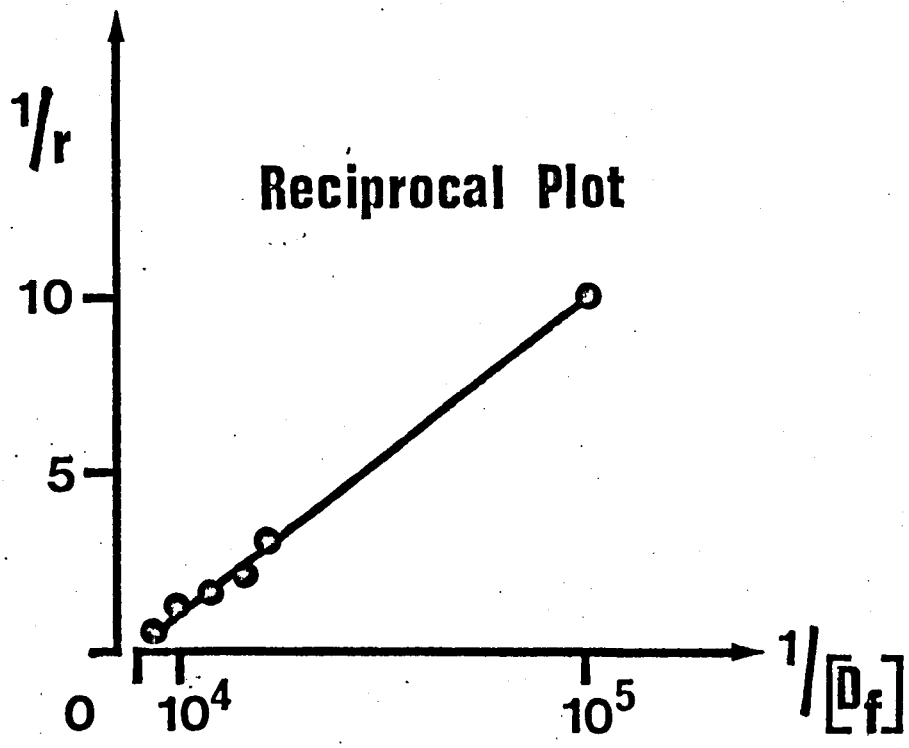
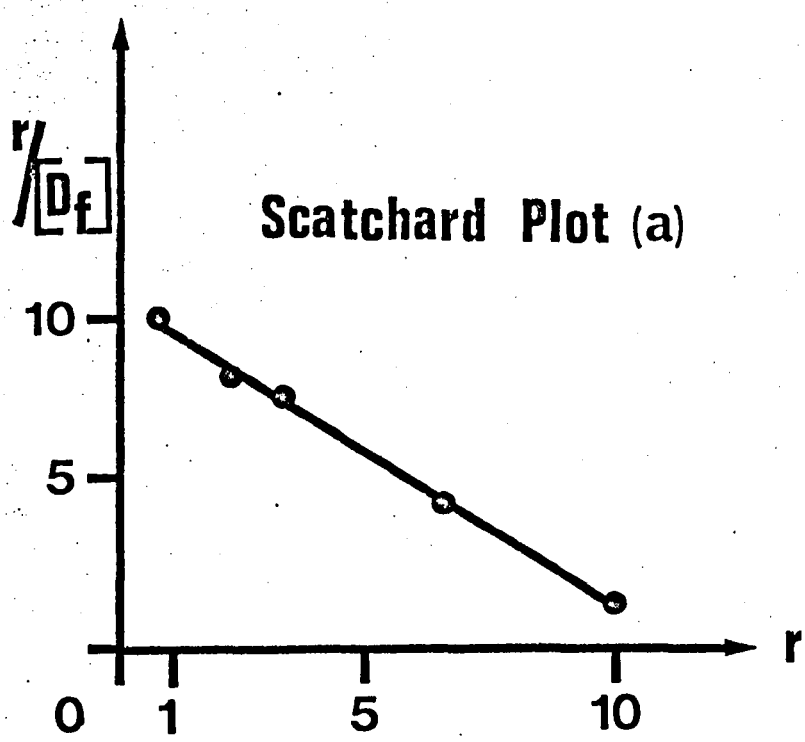


Figure 10 Different methods for the graphical representation of protein binding data.



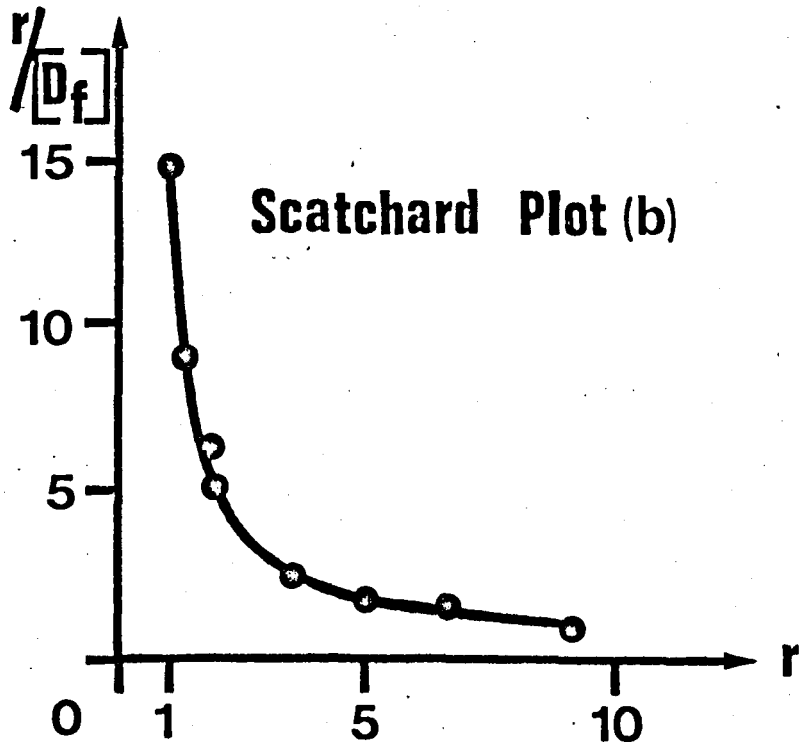


Figure 11 Scatchard representation of protein binding.

a. One class of binding sites

b. Two classes of binding sites

A plot of $\frac{(Db)}{(Df)}$ vs (Db) is independent of protein concentration and allows estimation of K from the slope. This plot will also be curved if more than one protein or class of binding sites are involved. This treatment is particularly useful when studying mixed protein systems, such as serum or plasma, when it is not known to which fraction the drug binds.

In all the above mathematical treatments of drug binding data it is assumed that the binding sites of a given class are independent of each other. While this may be true for many drugs, there are examples of co-operativity, e.g. the binding of oxygen by haemoglobin. Scatchard et al. (1950) have devised a rather complex expression to overcome non-ideality due to electrostatic interaction in the binding of charged molecules. However, most workers accept that it is adequate to report uncorrected data.

The Effect of Protein Binding on Drug Disposition and Pharmocological Activity

The ubiquitous nature of protein binding has been recognised and presumed to be a determining factor in drug disposition and effect (Martin, 1965; Levy, 1976; Booker and Darcy, 1973; Schoeman and Azarnoff, 1975). Most workers now accept that drug disposition and pharmacological activity is a function of unbound drug rather than total plasma levels.

There is also clear evidence that the distribution of certain drugs is controlled, at least partly, by protein binding. Studies in epileptic patients has indicated that the ratio of the C.S.F. to plasma concentrations for diphenylhydantoin (Lund et al., 1972; Paxton et al., 1977; Hanghton et al., 1975) phenobarbital and primidone (Hanghton et al., 1975) in the steady-state are about equal to their unbound concentrations in plasma. Further, there is a close correlation between the unbound drug in plasma and the concentration in erythrocytes (Borondy et al., 1973) and saliva (Svensmark et al., 1960) over a wide range in plasma concentrations. Also the steady state concentrations of nortriptyline in C.S.F. is only 3-11% of the steady-state plasma levels, which agrees well with the unbound plasma levels of about 6% (Borga et al., 1969).

The penetration of ampicillin and cloxacillin into synovial fluid of patients with osteoarthritis or rheumatoid arthritis has been examined (Howell et al., 1972). Both drugs diffused rapidly into synovial fluid, but the concentration attained differed appreciably and appeared to be related to the unbound concentration in plasma. Ampicillin is not highly bound and the concentrations in synovial fluid were similar to the total plasma level. Cloxicillin is about 95% protein bound, and unbound levels in plasma and synovial fluid were similar.

Linear correlations between the ratio of the concentration in erythrocytes to that in plasma and the percentage unbound drug in plasma for propranolol (Evans et al., 1973) and quinidine (Hughes et al., 1975) has been observed.

Of the first demonstrations of a relationship between unbound drug and pharmacological activity were the experiments of Anton, (1960). He studied the effect that sulphonamide binding to plasma proteins had on antibacterial activity using an equilibrium dialysis technique and demonstrated that only unbound dialysible drug mediated an effect. However, these were invitro experiments and the extrapolation to the invivo situation is tenuous. More convincing evidence was initially obtained in the work of Taylor, et al., (1954), who reported that recovery from thiopental anaesthesia was directly related to the decline of unbound drug in the blood of nephrectomised rabbits.

Most of the evidence for the pharmacological effect being due to unbound drug has been obtained by observing the response to alterations of protein binding induced by disease. Many pathophysiological states will cause a change of plasma albumin concentrations as a result of modifications in the synthesis rate, catabolic rate, or the distribution between the extravascular and intravascular spaces. Most disease states cause a lowering of plasma albumin levels (Table 4). The extent to which a drug is protein bound depends on the concentration of the drug, the association constant of the drug protein complex, the concentrations of the protein and the number of binding sites. These interrelationships are extremely complex, but there have been a number of reports showing the incidence of adverse drug effects is greater in patients with hypoalbuminaemia.

Unwanted central nervous system depression in patients on diazepam occurs in about 3% of patients with normal serum albumin levels. However, this can be as high as 9% in patients with low albumin levels (Greenblatt and Kock-Weser, 1974). There is a close correlation between unbound diphenylhydantoin in plasma and concentrations in C.S.F. (Lund et al., 1972; Paxton et al., 1977). Hypoproteinaemia has been reported to be associated with an increased incidence of adverse reactions to this drug in treated patients (Lunde et al., 1970; Boston Collaborative Drug Surveillance Programme 1973).

	Disease State	Reference	
A. Large Changes	Liver Disease	Skrede et al.,	1975
	Renal Disease	Jensen et al.,	1967
	Burns	Birke et al.,	1960
B. Small Changes	Surgery	Casten et al.,	1943
	Febrile Infections	Casey et al.,	1973
	Prolonged Rest or Immobilisation	Plantin et al.,	1971
	Pregnancy or Women on Oral Contraceptives	Gleichmann et al.,	1973
	Neoplastic Disease	Midler et al.,	1950
		Steinfeld,	1960
		Casey et al.,	1973
	Gastrointertinal Tract Disease	Rothschild et al.,	1972
	Chronic Bronchitis	Bonomo and d'Addabbo	1964
	Cystic Fibrosis	Strober et al.,	1969
	Ageing	Wallace and Whiting	1974

Table 4 Disease states and physiological conditions causing a reduction in serum albumin concentration

Patients with impaired renal function have a high incidence of adverse drug reactions. Adreason, (1973) has demonstrated decreased binding of salicyclic acid, acetylsalicylic acid, phenylbutazone, phenytoin, thiopental and sulphadiazine in patients suffering from acute renal failure. Although these patients had lower than normal serum albumin levels, upon correction of the data for albumin concentration, the binding capacity was still diminished. Reduced capacity and affinity has also been shown for a number of drugs in uraemic sera(Schoeman and Azarnoff, 1972; Reidenberg and Affrine, 1973; Steele et al., 1979).

The rate of certain elimination processes has also been considered to be a function of free rather than total drug concentration. In healthy individuals the glomerular filtration is an ultrafiltrate of plasma, and only free (unbound) drug is filtered. Kunin et al., (1959) has shown, for example, that the rate of renal excretion of several tetracyclines is inversely related to their extent of plasma protein binding.

Drugs may also be excreted by the liver into bile, and subsequently into the intestine. This process can result in enterohepatic cycling which may continue until elimination by metabolism, renal and faecal excretion is complete. The initial process in biliary excretion is the entry into liver cells (Combes, et al., 1956) which has been reported to be influenced by the degree of protein binding (Brauer and Pessotti, 1959; Priestley and O'Reilly, 1966). Lasser et al., (1962) observed that in a homologous series of labelled agents, the highly bound homologues were excreted in the bile, whereas the poorly bound members tended to be excreted in the urine. Knoefel (1965) suggested that certain highly bound drugs are excreted in bile because they are not available for glomerular filtration.

Drugs apparently diffuse passively into saliva, the transfer depending on lipid solubility and pKa. Also the extent of this transfer is related in some instances to the plasma protein binding e.g. a good correlation between free drug levels in plasma and drug levels in saliva have been reported for salicylates

(Graham and Rowland, 1972), digoxin (Huffman, 1975), tolbutamide (Martin et al., 1974), diphenylhdydantoin (Paxton et al., 1977).

Although the tone of this section is somewhat bland it is understandable. The effect of protein binding on drug distribution and pharmacological activity is not thought to be as important as it was considered to be a few years ago. It should nevertheless be emphasised that for particular drugs, e.g. diphenylhydantoin, knowledge of the degree and nature of the plasma or serum protein binding is a necessary prerequisite to sound drug therapy.

CHAPTER 2

MATERIALS AND METHODS

Materials:

All inorganic reagents and organic solvents were obtained from BDH Chemicals Limited unless otherwise stated.

Ponceau S was obtained from: G.T. Gurr Limited, London.

Salicylate, sulphadiazine, diphenylhydantoin and cyclophosphamide were purchased from Sigmia London Limited. Phenylbutazone was generously supplied by Geigy Pharmaceuticals and adriamycin by Farmitalia Products Limited, Barnet, Hertfordshire.

Methotrexate was obtained from the British Pharmacopoeia Commission Laboratory, Stanmore, Middlesex and Lederlie Limited, London. 7-Hydroxymethotrexate was kindly gifted by: Dr. D.G. Johns, National Cancer Institute, Bethesda, Maryland, USA, and rabbit antisera was kindly supplied by Dr. J.W. Paxton, the University of Auckland, Auckland, New Zealand. ^{75}Se- and ^{3}H- labeled methotrexate were supplied by the Radiochemical Centre, Amersham.

The Multimicro Concentrator (MMC) apparatus and Diaflo ultrafiltration membranes were obtained from Amicon Limited, Woking, Surrey.

RADIOIMMUNOASSAY OF METHOTREXATE

Radioimmunoassay Procedure

The assay procedure and quantities of reagents used are outlined in Table 5 .

Diluent Buffer:

0.05 M phosphate buffer, pH 7.4 containing 0.1% sodium azide and 0.1% bovine serum albumin.

Label:

^{75}Selenium - methotrexate (Fig. 12) specific activity (s.a.) 20 Ci mMol^{-1} in aqueous ammonia/20% ethanol. (v/v) was dissolved 1:1000 for use.

1st Antibody:

Rabbit antiserum, diluted 1:200 for use.

2nd Antibody:

Donkey anti-rabbit precipitating serum. Diluted 1:5 for use.

Standards:

Dissolved in diluent (Table 6). A typical standard curve is shown in Fig. 13 .

Quality Controls:

Dissolved in diluent (Table 7). Quality controls were set up immediately after the standards, after every twenty samples and again at the end of the assay.

Samples:

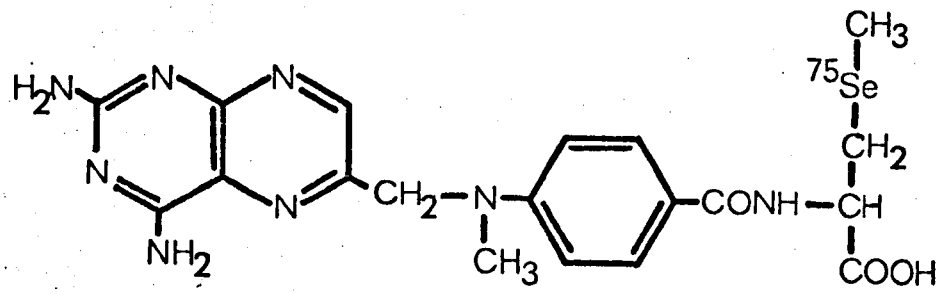
10 - 200 μl quantities were used in the assay or dilutions made depending on the concentration of methotrexate in the sample. An equivalent amount of normal human serum (N.H.S.) was added to the standard curve and quality controls, since the assay is sensitive to the presence of serum or plasma proteins (Fig. 14).

The standard curve, quality controls and patients' samples were done in duplicate. After the addition of all the reagents the LP3 tubes were mixed, without frothing, on a vortex mixer and incubated at 4°C for one hour (minimum period) then centrifuged at 4°C for 30 minutes at 800 g and the supernatant aspirated off. The tubes were counted on a Wallac gamma counter for 1-2 minutes to accumulate 10,000 counts in the total tubes.

The inter and intra assay precision of spiked samples are shown in Table 8 . and the precision of control samples estimated, without prior knowledge of the concentrations, over a period of one month are shown in Table 7 .

	Diluent Buffer	Sample	Label + NRS	1st Ab.	2nd Ab.
4 Total counts	-	-	50	-	-
2 Serum blanks	400	-	50	-	50
Standard curve	300	50 (St.)	50	50	50
Quality controls	300	50 (Q.C.)	50	50	50
Patients' samples	150-340	10-200 (Pat.Sam.)	50	50	50

Table 5 The radioimmunoassay procedure indicating the volume (µl) of reagents and patients samples used.



[75Se]MTX

Figure 12 The structure of ^{75}selenium methotrexate

Standards	pg/50 μl	0	10	20	40	60	100	200	400	600	1000	20000	40000
	ng/ml	0	0.2	0.4	0.8	1.2	2.0	4.0	8.0	12.0	20.0	40.0	80.0

Table 6 Concentration of solutions used to construct a standard curve

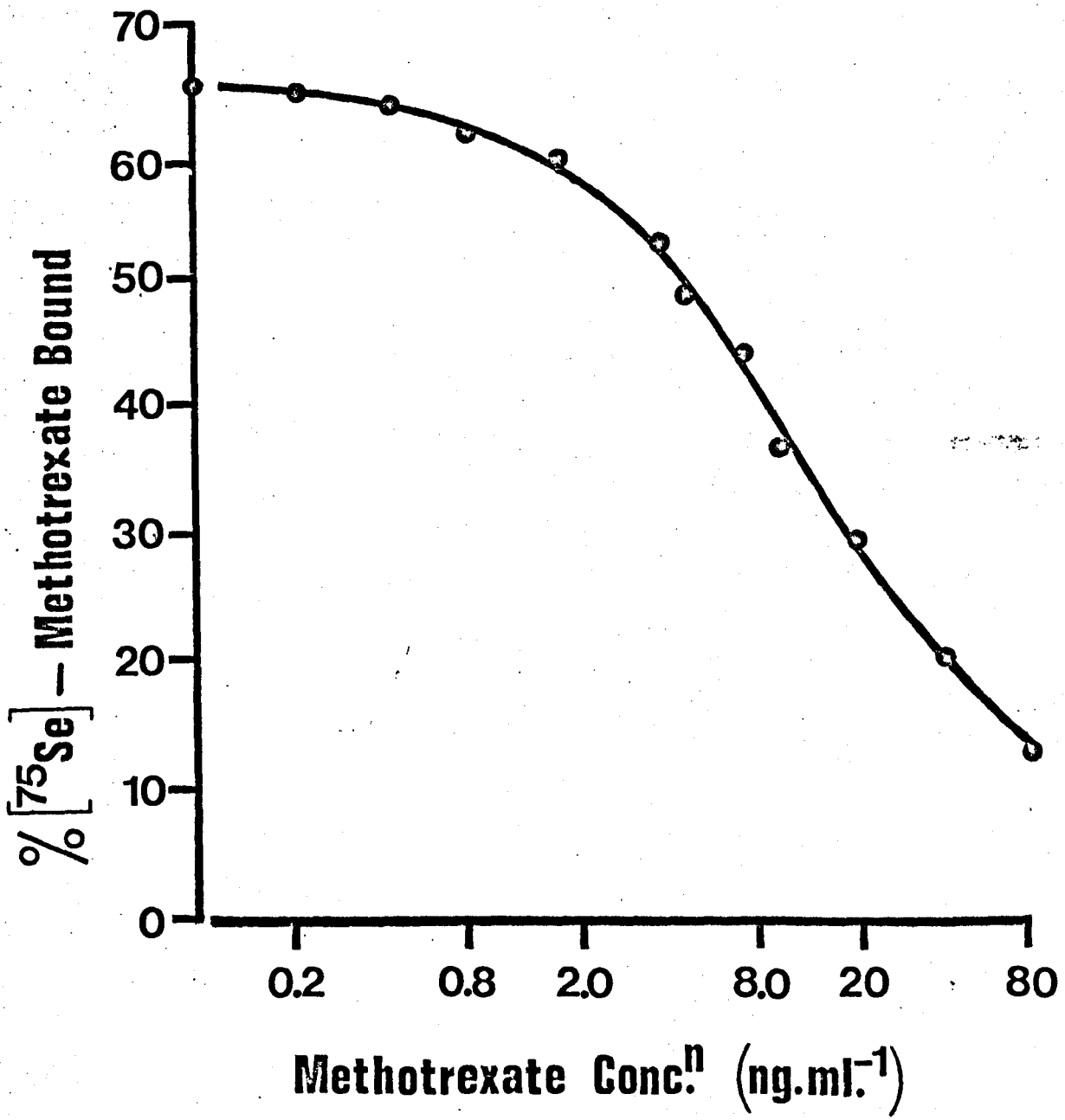


Figure 13 A typical radioimmunoassay standard curve

a.

		A	B	C
Quality controls	pg/50 l	75	750	2,250
	ng/ml	1.5	15.0	45.0

b.

	A	B	C
Inter-assay Precision (c.v.)	8.7	7.5	8.5
Intra-assay Precision (c.v.)	6.5	5.1	7.2

c.v. = coefficient of variation

Table 7 (A) The concentrations of quality controls
(B) Precision of quality controls measured over one month

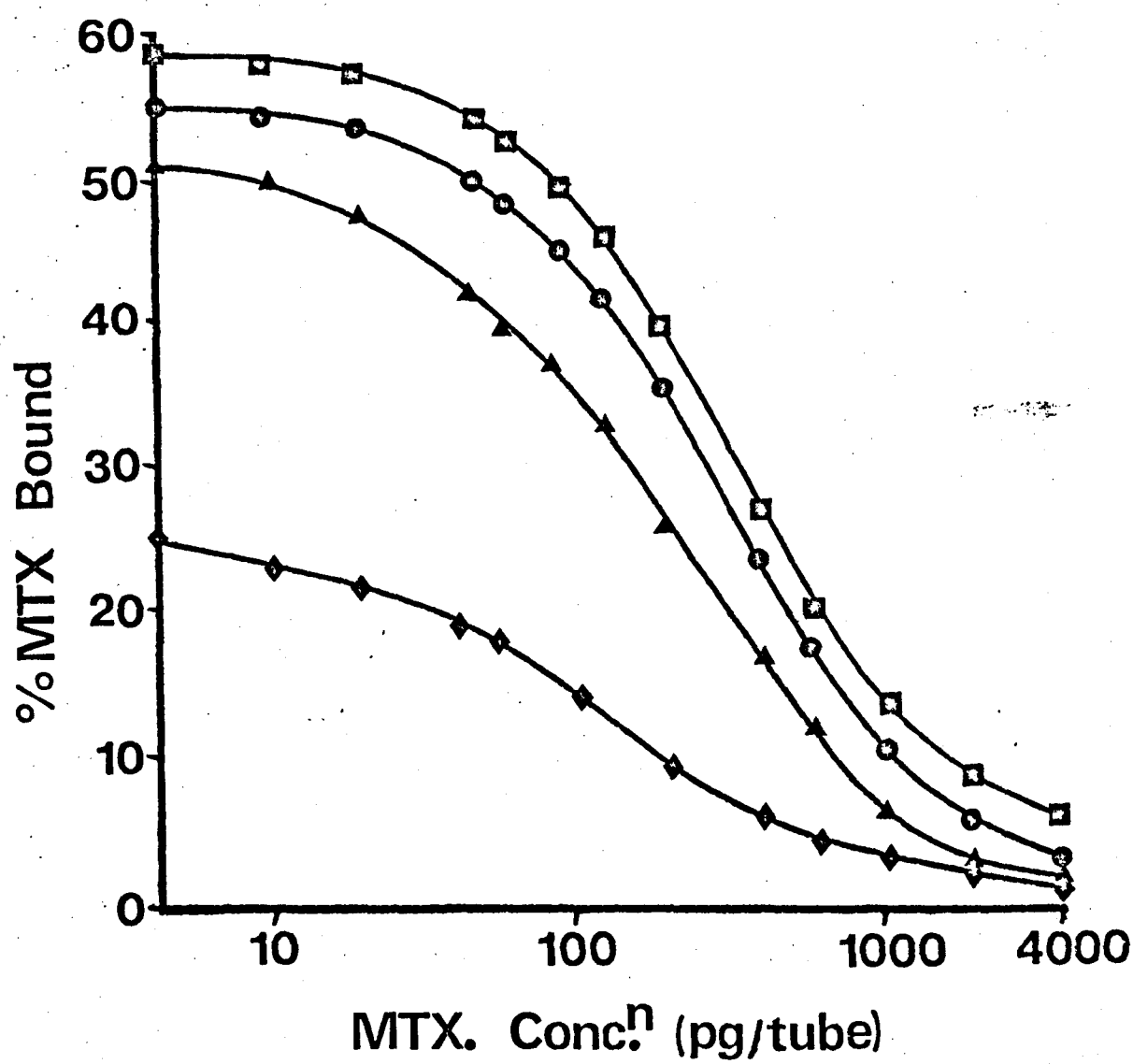


Figure 14 Effect of normal human serum (N.H.S.) on the binding of ^{75}Se-methotrexate to antisera

■: buffer only

●: 2% N.H.S.

▲: 10% N.H.S.

◆: 40% N.H.S.

		Intra Assay Precision			Inter Assay Precision		
N° of Tubes	(MTX) Added (Molar)	*(MTX) Measured (Molar)	C.V.	Recovery %	*(MTX) Measured (Molar)	C.V.	Recovery %
8	2.20×10^{-8}	2.38×10^{-8}	6.5	108	2.33×10^{-8}	8.7	106
8	2.20×10^{-6}	2.24×10^{-6}	3.6	102	2.20×10^{-6}	7.5	100
8	1.10×10^{-4}	2.08×10^{-4}	2.8	98	1.08×10^{-4}	8.1	98

Table 8 Methotrexate radioimmunoassay intra- and inter- assay precision

PROTEIN BINDING

Protein Binding by Continuous Ultrafiltration

The Amicon Multimicro Concentrator (MMC) apparatus (Fig. 15) was used to study the protein binding of MTX by continuous ultrafiltration. Serum, in quantities of 4 ml., were introduced into the M.M.C. chamber via filling ports using disposable plastic syringes. A known quantity of MTX was dissolved in phosphate buffered normal saline (pH 7.4) and placed in the reservoir. By locating the MMC equipment within an incubator at 37°C the protein binding experiments were performed at normal body temperature.

The MMC system was connected through a selector valve to a nitrogen cylinder, which provided the driving pressure for ultrafiltration. The tubing connecting the reservoir and ultrafiltration chamber were filled with drug solution. The selector valve on the MMC was opened to allow equilibration of the gas pressure in the chamber and reservoir. The selector valve was then immediately changed to connect the reservoir solution to the chamber, and continuous ultrafiltration commenced. Diaflo XM_{50} ultrafiltration membranes were used and the procedure was carried out at 40 psi which permitted a flow rate of ultrafiltrate from the chamber of 3 ml h^{-1}. Ultrafiltration volumes of 0.4 - 0.7 ml were collected into preweighed tubes and the exact volume determined by re-weighing to five decimal places. The temperature effects on the buffer volume at room temperature were found to be negligible. The serum within the chamber was continuously agitated by a magnetic stirrer mechanism during experiments. This not only facilitated mixing of MTX solution and serum, but prevented plaguing of serum proteins on the ultrafiltration membranes which would otherwise tend to occur due to lamina flow through the chamber.

In each collection period the concentration of free, total and bound MTX could be measured or calculated as follows:-

Let v_i = volume of the i^{th} ultrafiltrate in litres.

V = volume of the serum in the chamber in litres.

(R) = MTX concentration in the reservoir in Moles l^{-1}.

(M) = MTX concentration in the i^{th} collection in Moles l^{-1}.

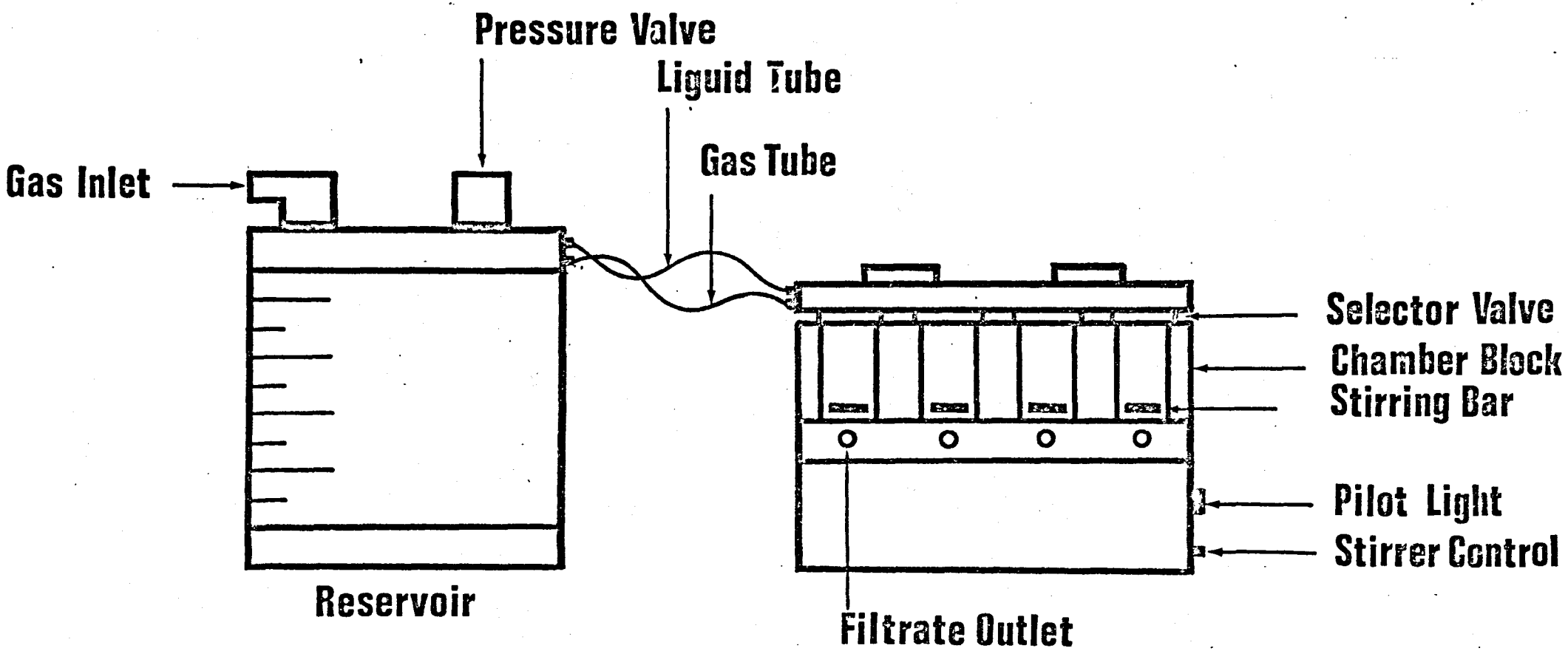


Figure 15 The Amicon Multimicro Concentrator (MMC) Unit

Since it is a closed system, the volume entering the chamber in the i^{th} period exactly equals the volume leaving the chamber ie (v_i)

$\therefore$ Moles entering chamber = v_i (R)

Moles leaving the chamber = v_i (M)

Moles retained in chamber = v_i (R) - v_i (M)

At the end of the i^{th} period the increase in total concentration in the chamber during the i^{th} period:

$$= \frac{1}{V} (v_i (R) - v_i (M)) \text{ Moles } l^{-1}$$

At the end of the nth period the increase in total concentration in the chamber:

$$= \frac{1}{V} \sum_{i=1}^{i=n} (v_i (R) - v_i (M)_i) \text{ Moles } l^{-1} \quad \text{----------1}$$

During the nth period the MTX concentration in the ultrafiltrate collected:

= Moles l^{-1} unbound in chamber

= (M)n

Therefore at the end of the nth period the moles l^{-1} bound:

$$= \frac{1}{V} \sum_{i=1}^{i=n} (v_i (R) - v_i (M)_i) - (M)_n \quad \text{----------2}$$

Thus, the continuous ultrafiltration technique allows in a single experiment a precise investigation of ligand-macromolecular interaction over a wide range of ligand concentration. A scatchard plot for MTX - protein interaction such as in Fig. 16 could be constructed from a single experiment from which an estimate of the binding parameters could be obtained.

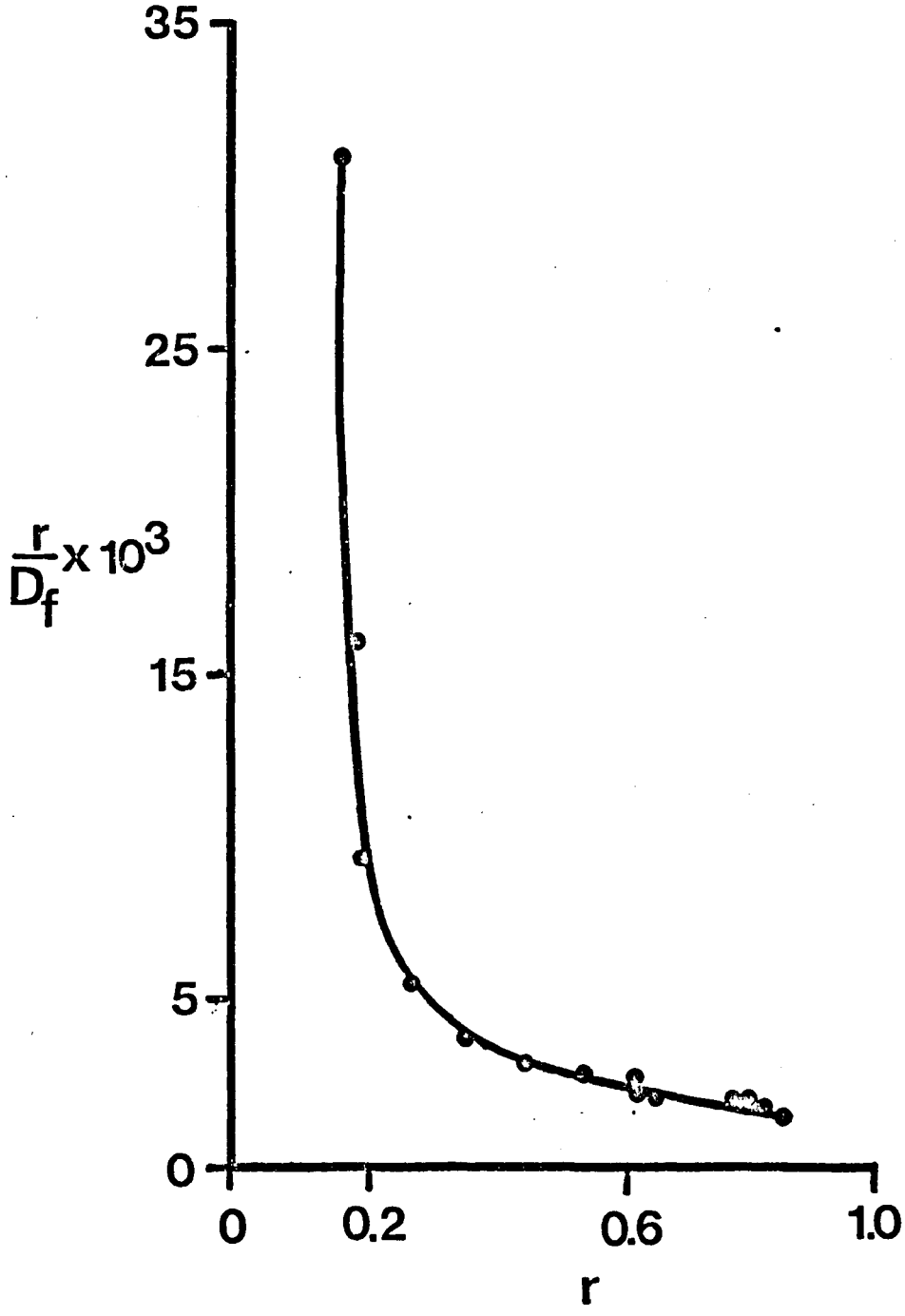


Figure 16 A typical scatchard plot for methotrexate
Df = concentration of free drug (Moles l^{-1})
r = Moles of drug bound per mole of albumin.

PHARMACOKINETIC AND STATISTICAL ANALYSIS

Pharmacokinetic Analysis

The following formulae were applied to the elucidation of kinetic parameters:

Biological half life $(t_{\frac{1}{2}}) = \frac{0.693}{\beta}$

Where β is the slope of the terminal phase of the concentration / time curve.

Area under the concentration/time curve (AUC) :

Linear trapezoidal method: $AUC_m^n = \sum_{i=m}^{n} \frac{(C_{pi+1} + C_{pi}) \cdot (t_{i+1} - t_i)}{2}$

Logarithmic trapeziodal method:

$$AUC_m^n = \sum_{i=m}^{n} \frac{(C_{pi+1} - C_{pi}) \cdot (t_{i+1} - t_i)}{\ln (C_{pi+1}/C_{pi})}$$

Where $(t_{i+1} - t_i)$ is the time interval between consecutive measurements of methotrexate concentrations (C_{pi} and C_{pi+1}).

The linear trapezoidal method of estimating AUC was used during the absorption phase after orally administered drug. The logarithmic trapezoidal method was used after intravenous administration or during the post absorption phase after oral dosing. The logarithmic trapezoidal method gives a better estimate of the area under curves with logarathmic decay, especially where large gaps between sampling points occur.

The area under the curve between the last data point and infinity is given by the expression: $AUC_r^{\infty} = \frac{C_{pr}}{\beta}$

Where C_{pr} is the concentration at the last data point on the concentration/time curve. Since this area is relatively small any error in its estimation would have a negligible effect on the overall area.

$$\therefore AUC_o \text{ (oral)} = \left[\sum_{i=m}^{n} \frac{(C_{pi+1} + C_{pi}) \cdot (t_{i+1} - t_i)}{2}\right] + \left[\sum_{i=n}^{r} \frac{(C_{pi+1} - C_{pi}) \cdot (t_{i+1} - t_i}{\ln (C_{pi+1}/C_{pi})}\right] + \frac{C_{pr}}{\beta}$$

and: AUC_o^{∞} (i.v.) = $\left[\sum_{i=m}^{r} \frac{(C_{pi+1} - C_{pi}) \cdot (t_{i+1} - t_i)}{\ln (C_{pi+1}/C_{pi})}\right] + \frac{C_{pr}}{\beta}$

Where m is zero time and n and r are later times.

Total body clearance $_{o-n}$ $(CL_{tot}) = \frac{\text{Dose}}{AUC_o^n}$

Renal clearance $_{o-n}$ $(CL_r) = \frac{\text{Amount of drug excreted in urine}_{o-n}}{AUC_o^n}$

Extra-renal clearance $(CL_{ex})_{o-\infty} = (CL_{tot})_{o-\infty} - (CL_r)_{o-\infty}$

Biliary clearance $(CL_{bil})_{o-n} = \frac{\text{Amount of drug excreted in bile}_{o-n}}{AUC_{o-n}}$

Where n is any time after intravenous drug administration.

Statistical Analysis

Results from each subject group were expressed as mean ± standard deviation (S.D.) using the equation:

$$S.D. = \sqrt{\frac{(x - \bar{x})^2}{n - 1}}$$

where x is any single value
$\bar{x}$ is the mean
n is the number of values in each group

The coeffecient of variation (C.V.) was obtained from the equation:

$$C.V. = \frac{S.D.}{\bar{x}} \times 100\%$$

Correlation analysis was performed using the Pearson's product moment correlation test in which the correlation coefficient is obtained from the expression:-

$$r = \frac{\sum(y - \bar{y})(x - \bar{x})}{\sqrt{\left[\sum(y - \bar{y})^2 \cdot \sum(x - \bar{x})^2\right]}}$$

Within subject differences were tested for significance using the Wilcoxon matched - pairs signed - ranks test e.g. comparing the AUC after 100 mg. bolus and after 25 mg. x 4.

The Mann - Whitney signed - ranks test for two independent samples was used to test for significance between different groups e.g. comparison of protein binding parameters of control and patient groups.

CHAPTER 3
RESULTS

SECTION 1 : Antibody Studies

1.1. The Cross-reactivity of 7-Hydroxymethotrexate

1.1. Cross-reactivity of 7-Hydroxymethotrexate (7-OHMTX)

Paxton et al. (1978) have measured the cross-reactivity of a number of similarly structured folates and other anti-metabolites with the antisera used in this methotrexate assay. Only dihydrofolic acid and folic acid showed any degree of cross-reactivity and then only to a negligibly small extent (0.03% and 0.01% respectively). However, the cross-reactivity of 7-hydroxy-methotrexate (7-OHMTX) had not been examined. It is known that the proportion of 7-OHMTX to MTX in serum and urine increases with time after administration of MTX (Jacobs et al., 1976). The possibility of cross-reactivity is therefore very real, particularly after high dose MTX and in samples collected more than 24 hours after the parent drug has been given. It is therefore of paramount importance to determine if such cross-reactivity does occur, and to what extent, since it could cause considerable error in the estimates of kinetic parameters.

7-Hydroxymethotrexate: The sample of metabolite weighed 2.9 mg. and was composed of 90% 7-OHMTX, 2% MTX and 7% other undescribed compounds.

Method: The specificity of the assay for MTX was calculated by adding increasing amounts of 7-OHMTX in place of non-radioactive MTX to the assay tubes and the percentage ^{75}Selenium (^{75}Se) label bound measured as described in the assay procedure. The cross-reactivity was then expressed as $(\frac{X}{Y} \times 100\%)$, where X is the molar weight of MTX and Y is the molar weight of 7-OHMTX, each required to produce 50% inhibition of specific binding of the label.

Results: The specificity of antisera using ^{75}Se - MTX is illustrated by the displacement curve shown in (Fig 17). The cross-reactivity of 7-OHMTX was 1.8%, therefore it would require a 55-fold higher concentration than MTX to produce a comparable displacement of ^{75}Se - label.

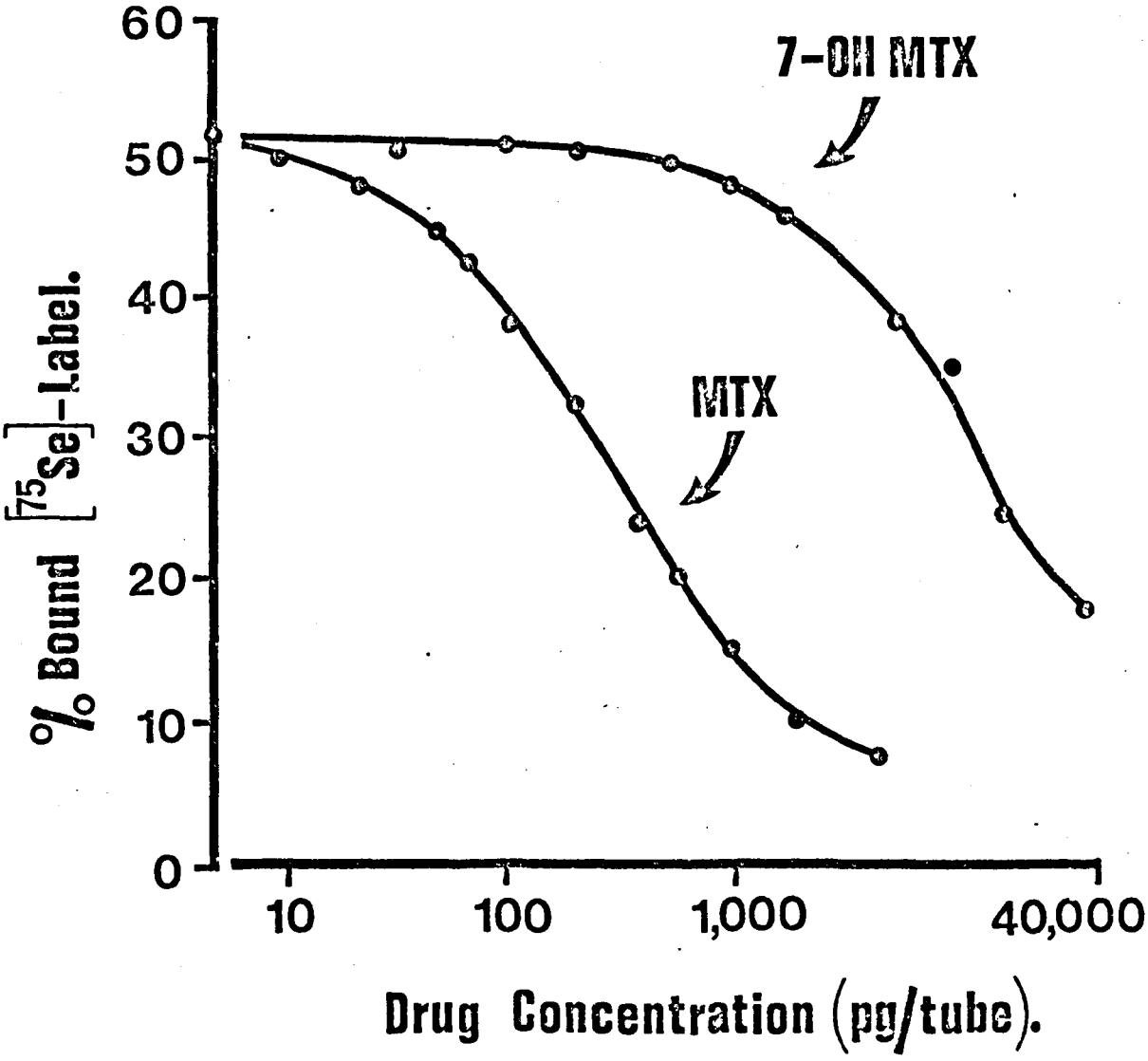


Figure 17 7-Hydroxymethotrexate (7-OHMTX) displacement curve

Discussion: The antisera used in the radioimmunoassay does not bind, to any great extent, the major metabolite (7-OHMTX) of MTX.

Considering the work of Paxton et al., (1978) the antisera would appear to be exceptionally specific. The high degree of specificity would permit the measurement of MTX in the presence of other naturally occurring folates. Further, it would permit the measurement of MTX in the body fluids of patients receiving high dose therapy with folinic acid rescue. Under this regimen considerable amounts of folinic acid and 7-OHMTX would be expected to co-exist with MTX in plasma and other body fluids.

The value obtained for the cross-reactivity of 7-OHMTX is very close to the percent MTX in the sample, being 1.8% and 2.0% respectively. It is therefore very likely that this degree of cross- reactivity simply reflects the assay of MTX in the sample. In any case it is clear that 7-OHMTX does not interfere significantly in this radioimmunoassay of MTX.

SECTION 2 : Methotrexate Disposition

2.1. : Intravenous Studies

2.1.1. Methotrexate in Tears and Saliva

Although MTX treatment schedules have been modified in an attempt to abrogate toxicity, oral toxicity and conjunctivitis are still frequently observed. In the past few years there has been considerable interest in the salivary excretion of drugs and a good correlation has been shown between salivary concentration and unbound fraction of drug in plasma or serum (Graham and Rowland, 1972; Kaysooko et al., 1973; Huffman, 1975; Martin et al., 1974; Paxton et al., 1977; Horning et al., 1977; Stephen and Speirs, 1976). The excretion of drugs in tears, however, is essentially unexplored.

The concentrations of MTX in mixed and parotid saliva and tears were therefore determined and related to the levels in serum particularly to the unbound fraction. The concentration of MTX in tears and saliva were also related to the incidence of ocular and oral toxicity.

Patients: Four patients with psoriasis and eleven patients with various forms of malignant disease gave their informed consent and participated in the study. Each was given either low dose (15-50 mg) or high dose (500 or 1000 mg) MTX intravenously, to be consistent with their normal therapy. A psoriatic was included in this study although she was given 25 mg. intramuscularly. Patient details are summarised in Table 9 . Patients on the high dose regimens, were also given folinic acid rescue (15 mg. folinic acid given 24 hours after drug administration followed by 5 mg. folinic acid q.i.d.).

Sample Collection: Serum: Blood samples in quantities of 5 ml. were obtained from an indwelling heparinised polyethylene catheter introduced into the left antecubital vein. Serum was removed by centrifugation after clot formation was complete.

Tears: Tears were collected on Schirmer test strips on which the sample volume (20-30µl) was determined by weighing. MTX was eluted using methanol (2 x 0.5 ml) which was subsequently evaporated to dryness and the MTX redissolved in 400 µl of buffer at 37°C. Standard solutions absorbed on the strips and eluted according to this procedure gave a good recovery (90-95%).

Patient	Sex	Primary Disease	Age (Y)	Alb (gl^{-1})	Dose (mg.)	% Unbound Drug	Oral Toxicity	Conjunctivitis
D.H.	M	Carcinoma Rib	57	33	1000	4.15	-	+
R.N.	M	Carcinoma Maxilla	51	35	1000	6.37	-	-
M.L.	F	Carcinoma Breast	60	37	1000	5.16	-	-
A.S.	F	Carcinoma Leg	90	29	500	3.73	-	-
R.P.	M	Carcinoma Penis	71	36	500	-	-	-
W.N.	M	Carcinoma Bronchus	61	39	50	2.77	-	+
A.C.	F	Carcinoma Breast	60	38	50	3.31	-	+
R.P.	M	Carcinoma Penis	71	36	50	5.73	-	+
M.G.	F	Carcinoma Breast	44	48	50	3.92	-	+
D.C.	M	Carcinoma Lung	56	38	50	4.61	-	+
B.H.	F	Carcinoma Breast	76	33	50	3.15	-	-
M.B.	F	Pustular Psoriasis	50	40	20	3.77	-	-
J.M.J.	M	Psoriasis	43	50	15	6.03	-	-
A.H.	M	Psoriasis	62	46	15	4.03	-	-
*A.M.	F	Psoriasis	70	43	25	11.74	-	-

Table 9 Summary of patient details, drug dose, percent unbound drug in serum and the incidence of oral toxicity and conjunctivitis

500 - 1000 mg : i.v. infusion over 12 h.

15 - 50 mg : i.v. injection

25 mg : i.m.

Parotid Saliva: Parotid saliva was collected using a parotid cup (Stephens and Speirs, 1976), salivary secretion being stimulated with citric acid (0.5 ml of 1% solution). The pH of the parotid saliva was checked using indicator paper to determine whether any citric acid was contaminating the sample. Approximately 1 ml. samples were collected.

Mixed Saliva: Samples of about 1 ml. in volume were collected by direct expectoration into plain tubes.

The patient's mouth was washed out with water and swabbed dry before the collection of each sample. Mixed saliva was collected and after reswabbing the parotid cup was placed in position. Blood, tears and parotid saliva were then obtained simultaneously. All sampling was completed within 3-5 minutes. Samples were collected at appropriate intervals from zero time until between 24 and 30 hours after initiation of therapy. All specimens were kept at -20°C. until assayed.

Methods: Protein binding was determined by continuous ultrafiltration as described previously. A reservoir concentration of 12 μmol.l^{-1} was employed, which enabled the study of binding over the range 1-30 μmol.l^{-1}. Adjustments in drug concentration within this range were performed stepwise yielding ten to fourteen estimates of protein binding, each pertaining to a different drug concentration.

Results: Soon after administration, MTX was detectable in tears and both parotid and mixed saliva (Fig.18:19)Regression analysis showed a close correlation ($p<0.001$, $r=0.714$) between MTX levels in tears and serum (Fig. 20). Parotid saliva and serum MTX also showed a close correlation ($p<0.001$, $r=0.557$, Fig.21). No significant correlation was found between MTX concentrations in mixed saliva and serum ($p<0.1$, $r=0.232$). The ratio of MTX level in tears: level in serum was 0.047:1, while the ratio of MTX level in parotid saliva : level in serum was 0.003.1. The protein binding was found to be 95.11% $\pm$ 2.26 (s.d.) and remained constant over the range 1 -30 μmol l^{-1}.

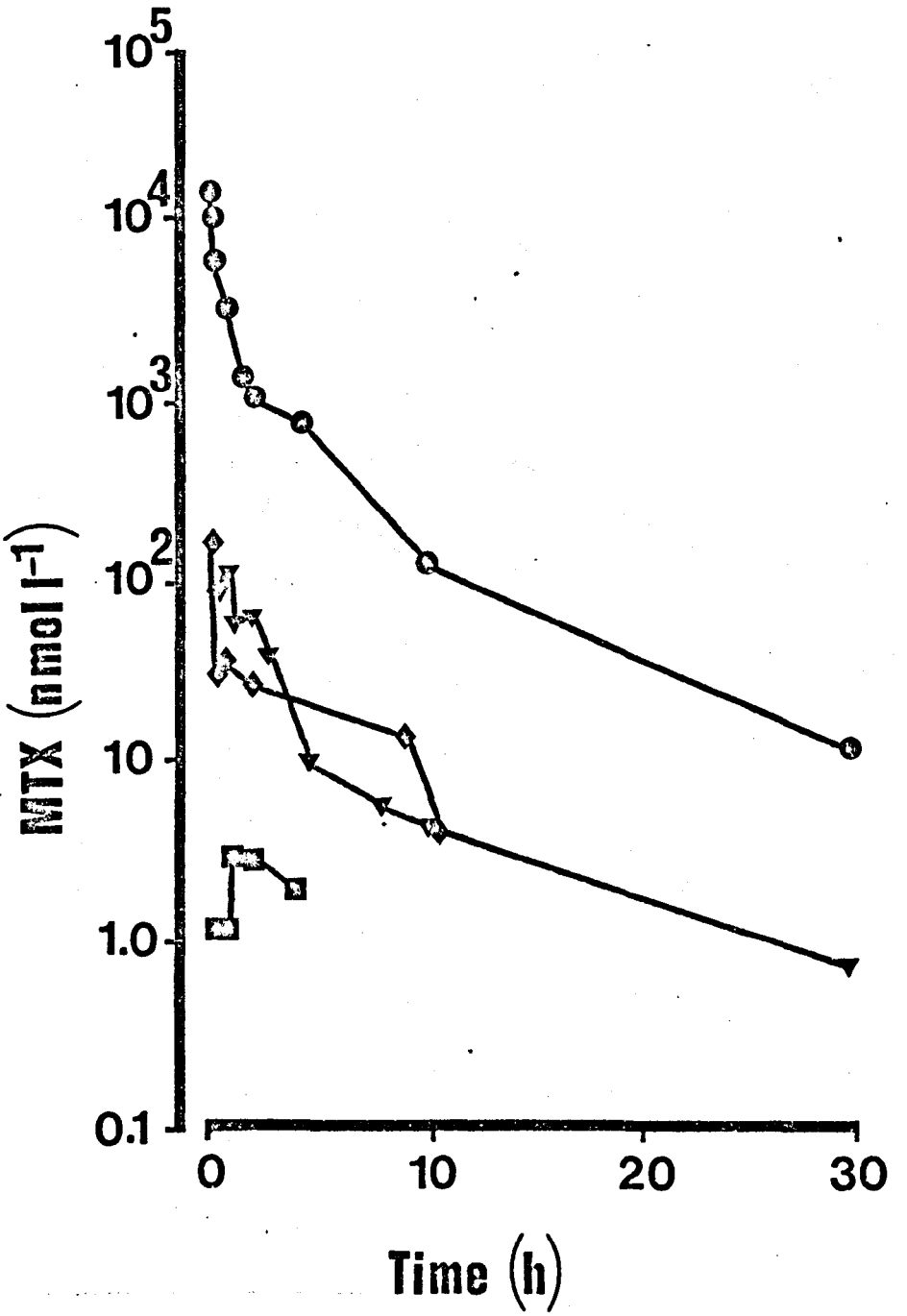


Figure 18 Serum (●), tear (▼), parotid (■) and mixed (◆) salivary methotrexate (MTX) concentration time curves in patient A.C. after a single 50 mg. i.v. injection

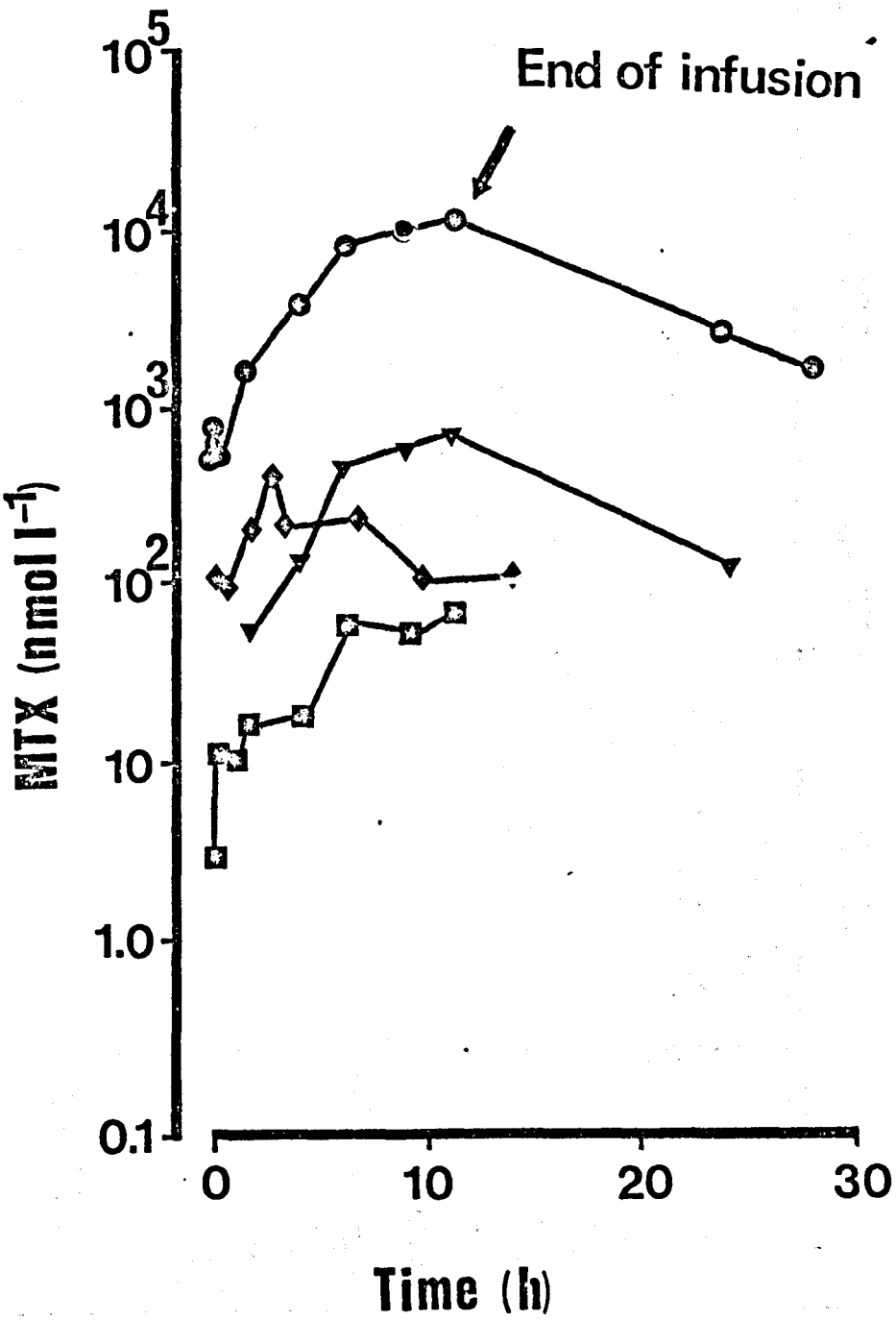


Figure 19 Serum (●), tear (▼), parotid (■) and mixed (◆) salivary methotrexate (MTX) concentration time curves in patient A.S. following a single 500 mg. i.v. infusion

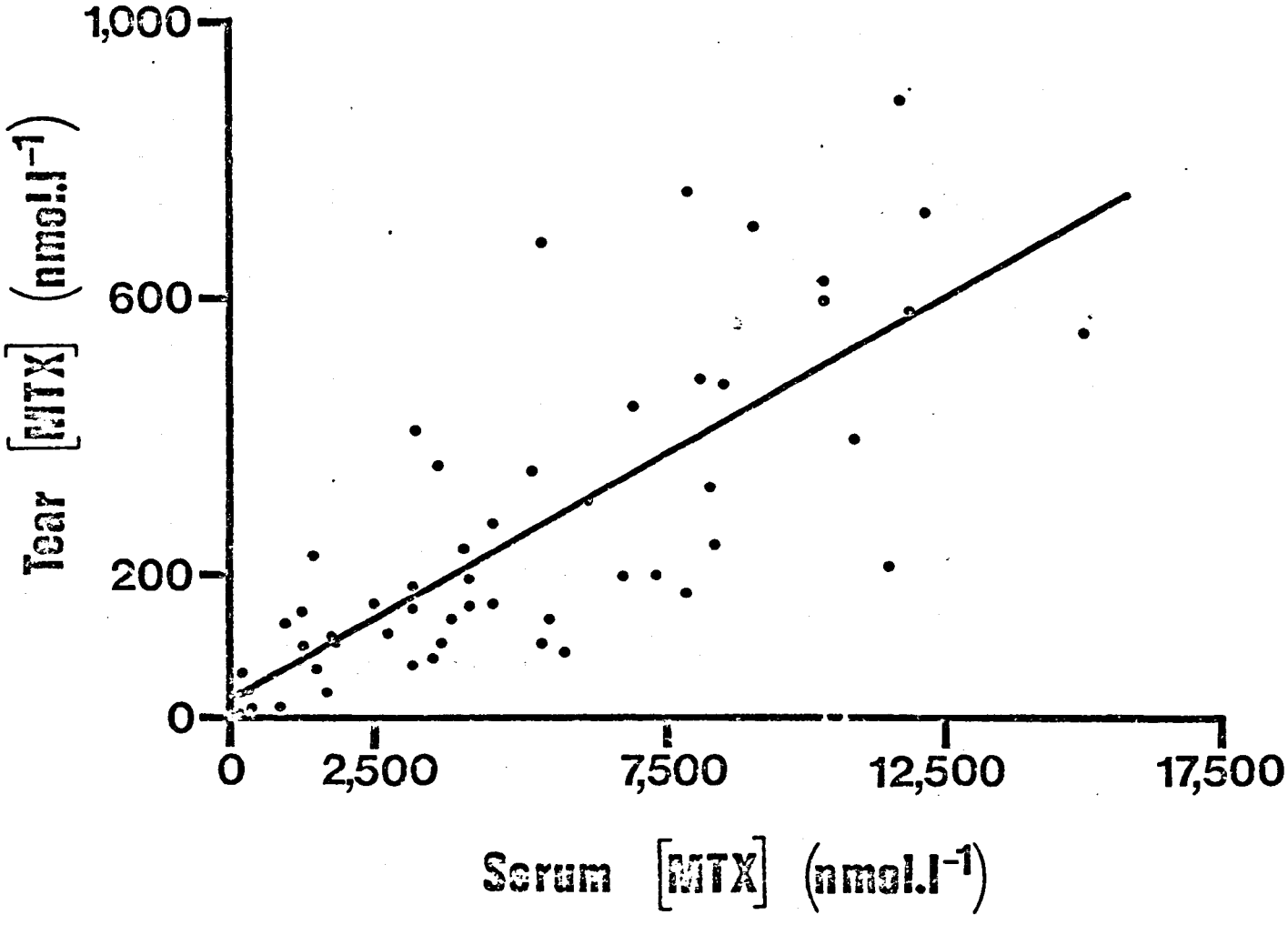


Figure 20 Relationship between tear and serum levels of methotrexate (MTX) in eleven patients ($p < 0.001$; $r = 0.714$)

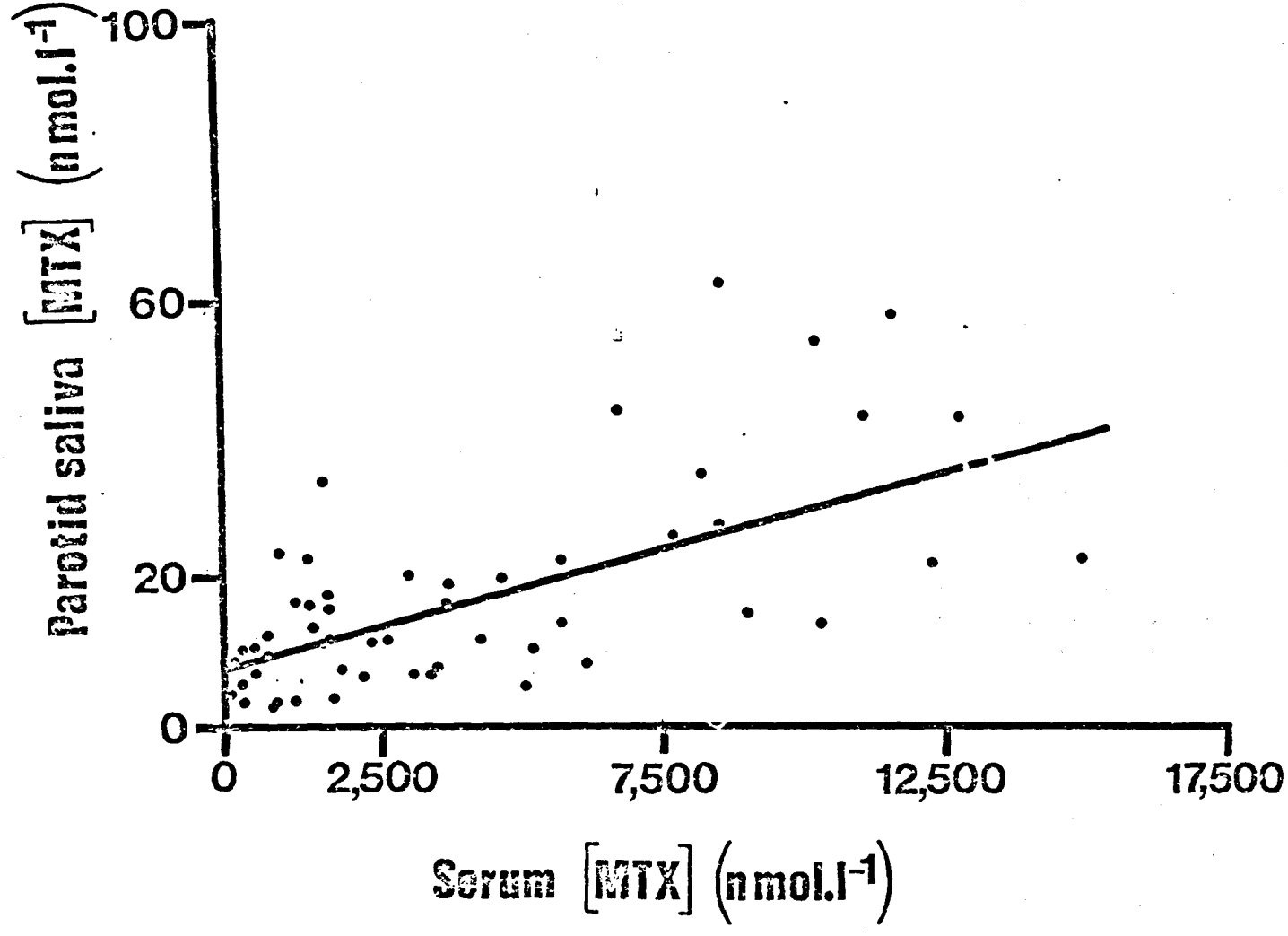


Figure 21 Relationship between parotid salivary and serum levels of methotrexate (MTX) in eleven patients ($p < 0.001$; $r = 0.557$)

No oral toxicity was observed in any of the patients during this study but a number showed signs of conjunctivitis. Table 9 shows the dose of drug given, the unbound drug level in serum and the incidence of ocular toxicity (viz. conjunctivitis). Having established that the ratio of MTX levels in tears to unbound drug levels in serum is very near unity, no relationship between tear concentrations of MTX and the incidence of conjunctivitis could be demonstrated. The incidence of conjunctivitis did not increase with increase in serum concentration even although higher levels of MTX were observed in tears.

2.1.2 Methotrexate in Cerebrospinal Fluid (C.S.F.)

The concentration of a number of drugs in C.S.F. correlate well with the unbound drug in serum (Hanghton et al., 1975; Borga et al., 1969; Paxton, et al., 1977). Since central nervous system toxicity in children, on high dose MTX chemotherapy, is occasionally observed (Wang, et al., 1976) a non-invasive method of assessing C.S.F. concentrations would be extremely useful in monitoring therapy.

The concentration of MTX in C.S.F. was measured and related to the levels of drugs in plasma and mixed saliva.

Patients and Methods: Nine children, aged between 3 - 12 years, on high dose MTX for leukaemia relapse were observed. This therapy consisted of 500 mg. m^{-2}, a third of which was administered as an intravenous bolus injection (over three minutes) and the remainder by infusion over eight hours. A few blood samples were taken during and after drug administration and C.S.F. withdrawn via an Ommaya tube, 1 hour after the bolus injection. Mixed saliva was collected by direct expectoration, immediately after blood was withdrawn. The collection of parotid saliva was found to be impractical and no attempt was made to impose the collection of tears. Five of the children were observed on two separate occasions.

Results: A typical concentration-time profile for plasma and mixed saliva is shown in Fig.22 . No significant correlation was found between either the concentration in mixed saliva and plasma ($p < 0.05$; $r=0.55$) or the level in C.S.F. and plasma ($p < 0.1$; $r=0.40$). However, there was a significant correlation between the concentration in mixed saliva and the concentration in C.S.F. ($p < 0.001$; $r=0.91$, Fig.23). The concentration of MTX in C.S.F. was approximately 0.1% of that in plasma. Similarly the concentration in mixed saliva was in the region of 0.1% of the level in plasma. Since there was a significant correlation between the concentrations in C.S.F. and mixed saliva, the ratio of MTX concentration in C.S.F. to that in mixed saliva was 0.93 : 1.0 .

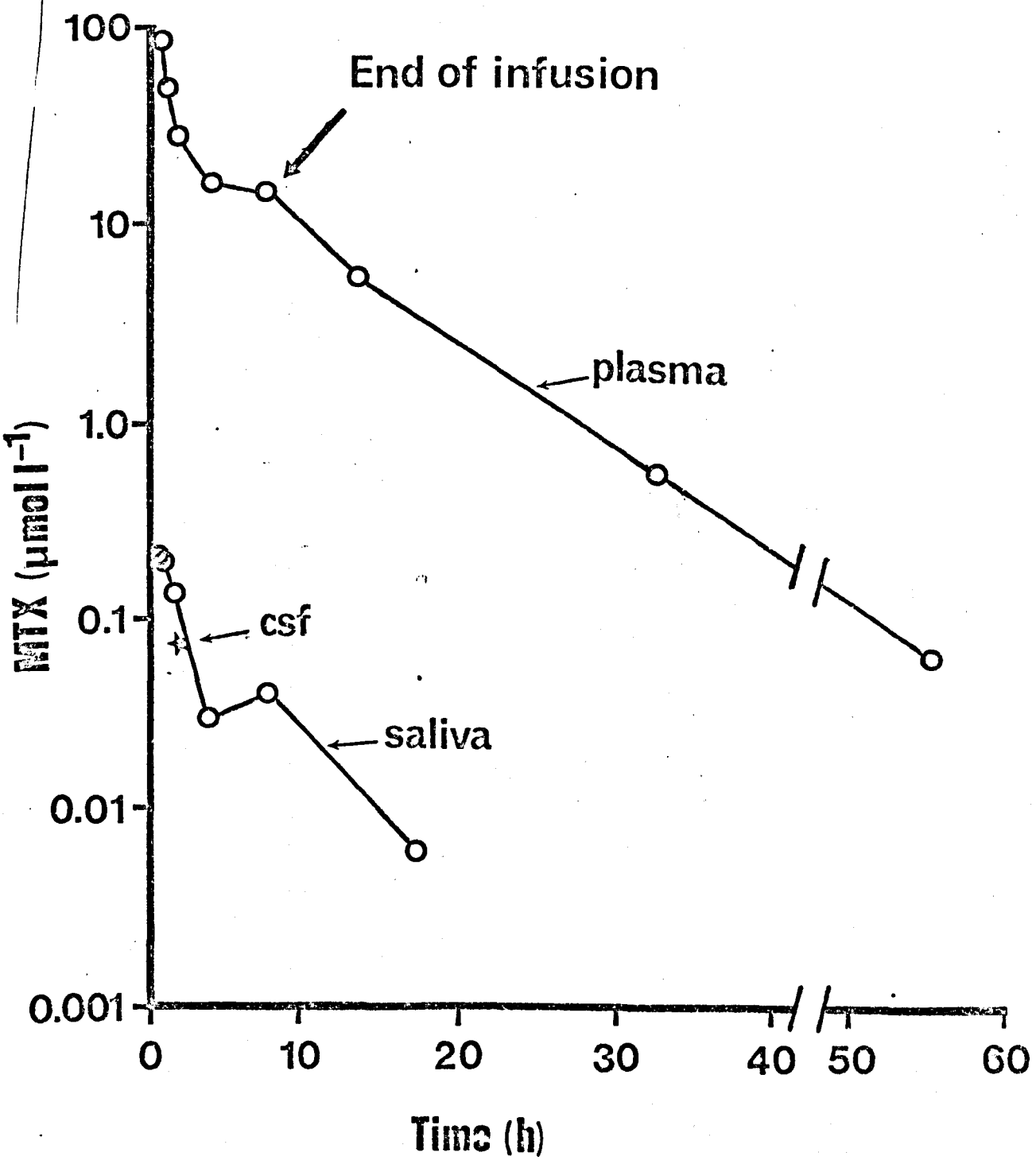


Figure 22 A typical concentration time curve showing methotrexate levels in plasma; mixed saliva and CSF.

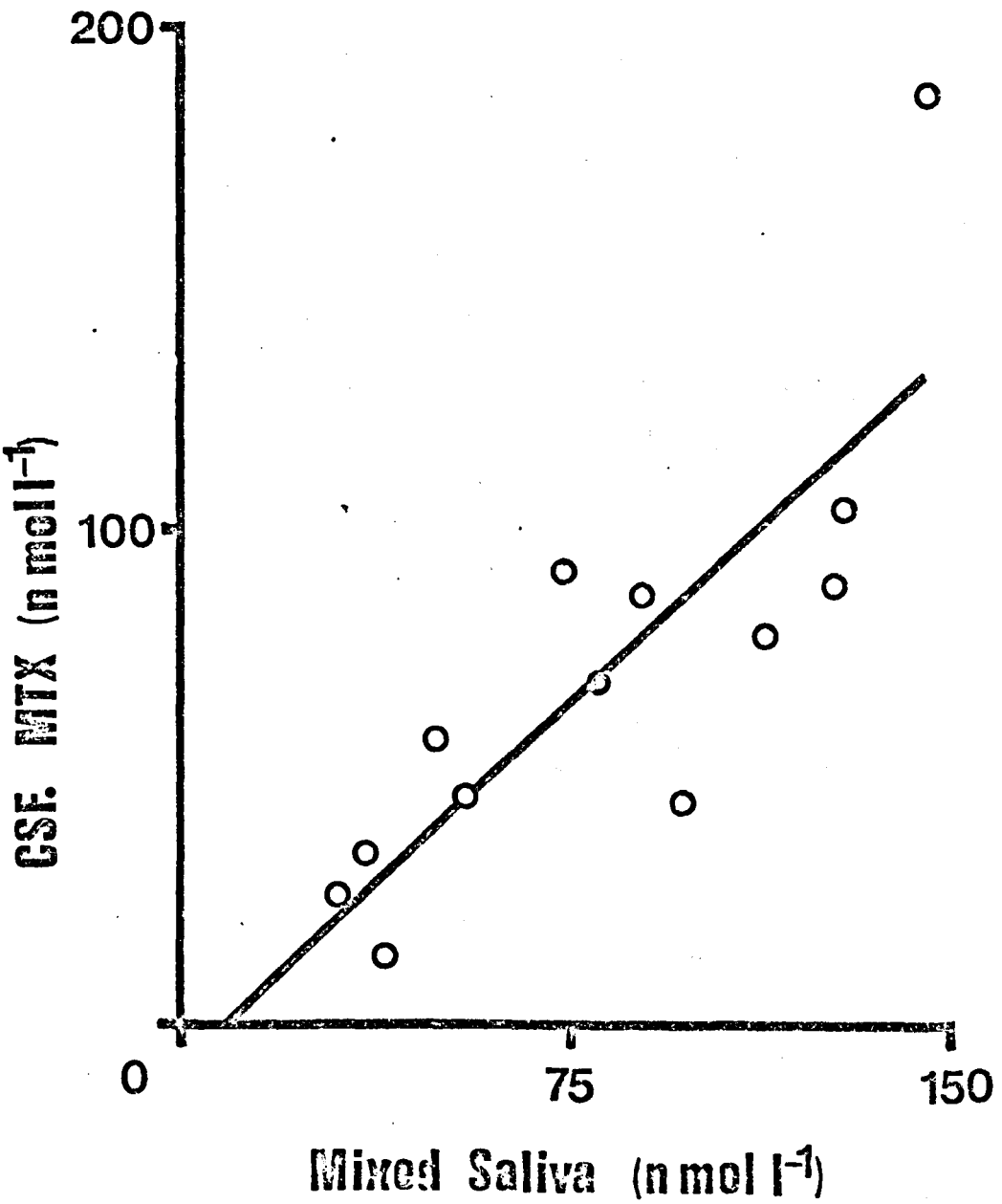


Figure 23 Relationship between CSF and mixed salivary levels of methotrexate. ($P < 0.001$: $r = 0.91$)

Discussion: It has been possible to demonstrate, in patients receiving MTX, significant linear correlations between drug concentrations in tears, serum and parotid saliva. Since the mean unbound fraction of MTX is 4.89%, it is likely that free drug in serum is in passive equilibrium with drug in tears. Furthermore, there is a predictable relationship between MTX in serum and drug in parotid saliva. However, the drug levels in parotid saliva were considerably lower than levels in tears. A possible explanation for this would be that MTX secretion is dependent on the rate of salivary flow. However, in view of the very low concentrations in parotid saliva, it is more likely that MTX is secluded from the secretions of the parotid gland.

There are a number of parameters which influence the partition of drugs between saliva and serum. These include the pKa value, the pH of the two fluids and the lipid solubility (Linde,, 1932; Glynn and Bastain, 1973; Feller and le Petit, 1977). Feller and le Petit (1977) have pointed out that considerable differences in concentration may arise between plasma and saliva due to different storage of the dissociated part, according to the pH-partition theory, if the pKa of an acid drug is lower than 8.5. Since the pKa values of MTX are 4.8 and 5.5 it would be expected from this concept, that MTX would be retained in the plasma compartment. Also drugs with low lipid solubility measured by the n-octanol/water partition coefficient, do not permeate lipid membranes well. Again from this theoretical approach it would be unlikely that MTX would have a 1:1 ratio with unbound drug in plasma since MTX is a highly polar molecule with a log P octanol of - 1.85 (Leo et al., 1971).

Although physico-chemical processes may determine a partition of MTX between serum and saliva, very little information is available concerning the factors responsible for this phenomenon in tears. Indications to study drug concentrations in tears are few, therefore one would hesitate to extrapolate these observations to other compounds. Ocular toxicity did not correlate with drug levels in tears. Higher concentrations of MTX were present in tears of those patients receiving the high

dose infusions and only one patient showed signs of conjunctivitis in this group. However, these patients were 'rescued' from toxicity with folinic acid whereas the patients given low doses were not. In contrast, the group given 50 mg. by intravenous injection, conjunctivitis was evident in all but one patient, at 7-14 days after drug administration. At this time no MTX could be detected in tears, but persistence of low residual levels could contribute to the observed ocular toxicity when folinic acid is not given.

No advantage would be served by the prediction of serum concentrations from that found in tears and saliva. The collection procedures are time-consuming and require special devices and materials which would be quite inappropriate under clinical conditions. Further, the collection of these fluids, particularly tears, would be unsuitable in children, where development of non-invasive and painless techniques for monitoring drug levels is most desirable. Further serum levels of MTX cannot be used to predict C.S.F. concentrations in children. However, a highly significant correlation was observed between the concentrations in mixed saliva and C.S.F. After high intravenous doses an estimate of C.S.F. concentrations could be made from that measured in mixed saliva obtained by simple expectoration. This could have great potential in determining not only possible central nervous system toxicity due to high C.S.F. levels of MTX, but also distinguishing between meningeal relapse and toxicity. One patient was excluded from the study due to overt oral toxicity with bucal ulceration. The source of salivary MTX in this patient was uncertain since the saliva was more cloudy than normally observed and some samples were flecked with blood. Salivary levels were six times higher than the C.S.F. concentrations in this patient. This illustrates one limitation of predicting C.S.F. levels from that in saliva, since oral toxicity may alter the ratio due to presence of blood or to increased

permeability of the mucus membrane resulting from desquamation of epithelial cells. Since there is insufficient data at present to determine the confidence with which one could predict the levels of MTX in C.S.F. from that measured in a single mixed salivary sample taken at random, these results should be considered with caution. The data does, however, indicate the need for further study since mixed saliva samples can be collected with ease and could provide a simple means of monitoring children on high dose MTX shhedules without causing the stress involved in the withdrawal of C.S.F.

2.1.3. Distribution of Methotrexate between Plasma & Erythrocytes

Apart from a few reports (Bobzien and Goldman, 1972; Rothenberg, et al., 1976) little attention has been focused on the distribution of MTX between erythrocytes and Plasma. There have, in particular, been no studies of MTX levels pertaining in erythrocytes in vivo.

The specificity and sensitivity of the radioimmunoassay has enabled levels of MTX in serum, whole blood and erythrocytes to be measured up to a time after administration when serum concentrations have been hitherto undetectable.

The changes in MTX concentration with time in plasma, whole blood and erythrocytes were monitored during and after high dose intravenous infusion.

Patients: Three patients with various forms of malignant disease were given 250 mg. MTX by intravenous infusion over 12 hours as part of their regular chemotherapy. In one patient the course of drug levels was observed on two separate occasions with an interval of one month. On the first occasion this patient had no previous history of chemotherapy, but, the other two patients had been receiving cytotoxic therapy for a few months. Folinic acid 'rescue' was a routine part of the chemotherapy received by these patients, and comprised 15 mg. folinic acid given intravenously 24 hours after initiation of therapy followed by 5 mg. folinic acid q.i.d.

Sample Collection: Whole Blood: Heparinised blood specimens of 10 ml. were withdrawn at appropriate intervals from zero time (pre-treatment sample) until between 8 and 16 days after administration. About 3-4 ml.aliquotes of whole blood were haemolysed by freezing and thawing, and in some specimens this was aided by dilution in distilled water.

Erythrocytes: The erythrocyte fraction was prepared by the following method. Immediately blood was withdrawn the packed cell volume (P.C.V.) was found for each specimen of blood.

A 1.0 ml. quantity of blood was then centrifuged and the plasma withdrawn. The volume of the packed cells was made up to 2.0 ml. with normal saline and the tube mixed thoroughly, but gently, by repeated inversion. The specimen was then recentrifuged at 800 g. for ten minutes. The supernatant was removed without disturbing the erythrocyte pellet and discarded. This washing procedure was repeated twice, and when the last supernatant was removed the volume was made up to 1.0 ml. with normal saline. After mixing thoroughly a second P.C.V. measurement was found. The specimen was again centrifuged, the supernatant discarded and the volume again made up to 1.0 ml. with distilled water to cause haemolysis. The washing procedure and centrifugation were performed at between 0° - 4°C. The difference between the pre- and post- washing P.C.V. allowed a correction for loss of erythrocytes in the procedure to be made. Haemolysis was further encouraged by dilution in distilled water immediately before the samples were assayed.

Plasma: The plasma fraction was removed from the remaining sample of whole blood after centrifugation. After re-centrifugation the plasma was carefully removed from the tube leaving 200-300 µl. to be discarded. This procedure was undertaken to ensure that no erythrocytes were carried over from the initial separation.

All specimens (haemolysed whole blood, haemolysed erythrocytes and plasma) were stored at -20°C. until assayed.

Methods: MTX concentrations were measured by a specific and sensitive radioimmunoassay (Paxton, et al., 1978) employing ^{75}Se-label.

The biologic half life of MTX in plasma was determined from the slope of the log/linear regression line (least squares fitting) drawn through the terminal portion of the serum concentration/time curve.

The increase in MTX concentration in erythrocytes with time observed when patient J.M. was studied on the first occasion was determined from the log/linear regression line (least squares fitting) drawn through the data from day 2 to day 16. This was used to determine the maximum MTX level attained on day 16.

Results: The concentration in the three fluids increased with time, reaching a peak at the end of infusion. The concentration in plasma and whole blood followed a similar pattern with the plasma levels consistantly higher than that in whole blood. The ratio of peak concentration in whole blood : concentration in plasma varied between 0.82:1 and 0.56:1. The concentration peak in erythrocytes at the end of infusion was very much lower than in serum, the peak concentration in erythrocytes : peak concentration in plasma varying between 0.03:1 and 0.01:1.

Immediately after completion of the infusion the MTX concentration in all three compartments fell sharply but the mean plasma biologic half life ($t_{\frac{1}{2}}$) measured over the terminal portion of the concentration time curve was 43.0 h. ± 6.22 (S.D.), The concentration in plasma fell below the limit of sensitivity of the assay between days 8 and 10. As this time was approached the concentration was much greater in the erythrocytes and whole blood. The concentration of MTX in erythrocytes tended to plateau and did not have an apparent elimination phase. At day 8 the erythrocyte concentration : concentration in plasma ratio, varied between 145.50:1 and 18.75:1 and the whole blood : plasma concentration ratio varied between 20.80:1 and 4.19:1. The individual data is summarised in Table 10. And Fig. 24 and 25

In one patient (J.M.) the course of drug levels was observed on two occasions with an interval of one month. On the first occasion the patient had no previous history of chemotherapy and there was a general increase in erythrocyte MTX concentration starting on day 2, after the initial drop at the end of the infusion, which continued until day 16 (when drug monitoring was stopped). The concentration increased from 46.78 $nmol.l^{-1}$ at 46 h. to 185.0 $nmol.l^{-1}$ at 381 h. This increase in concentration persisted at a time when plasma levels were decaying and even after the levels had become undetectable. The peak whole blood level : peak plasma level ratio, was 0.82:1 and the peak erythrocyte level : peak plasma level ratio was 0.03:1.

	Peak Ratios		Plateau Radios		Plasma
Patient	W.B. : P	R.B.C. : P	W.B. : P	R.B.C. : P	$t_{\frac{1}{2}}$ (h)
J.M. (1)	0·82	0·03	20·80	145·50	36·0
J.M. (2)	0·63	0·01	7·33	34·50	40·0
J.C.	0·56	0·01	12·73	18·75	46·0 h
M.K.	0·75	0·01	4·19	19·45	50·0

Table 10 Peak and plateau ratios for the individual patients.

Figure 24 The concentration time curves for methotrexate in plasma, whole blood and erythrocytes for patient J.M.

Figure 25 The concentration time curves for methotrexate in plasma, whole blood and erythrocytes for patients J.C. and M.K.

Key symbols : ● serum
■ whole blood
▲ erythrocytes

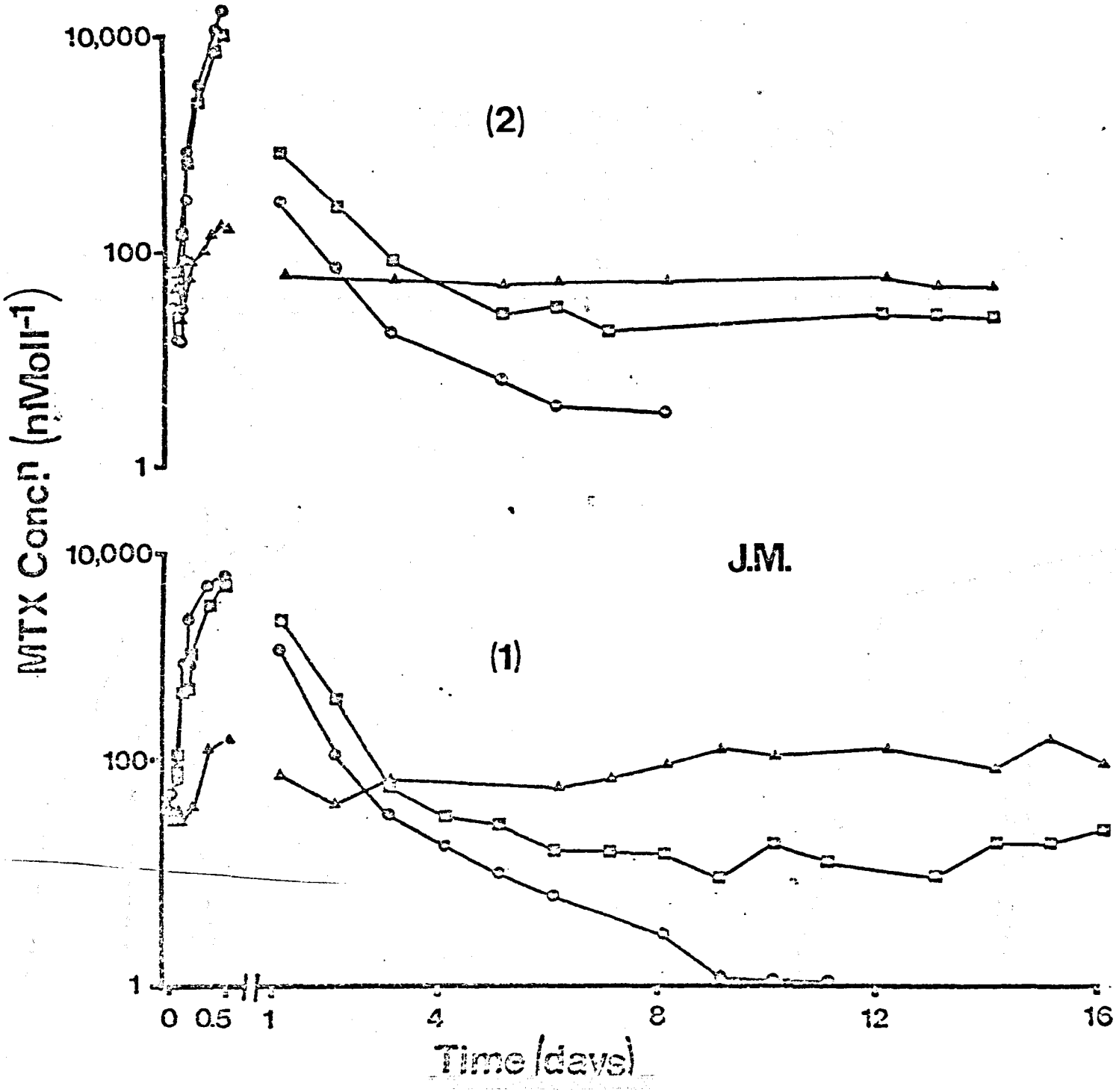

10,000
100
1
(2)
MTX Concn (nMolI⁻¹)
J.M.
10,000
100
1
(1)
0 0.5 1 4 8 12 16
Time (days)

FIGURE 24

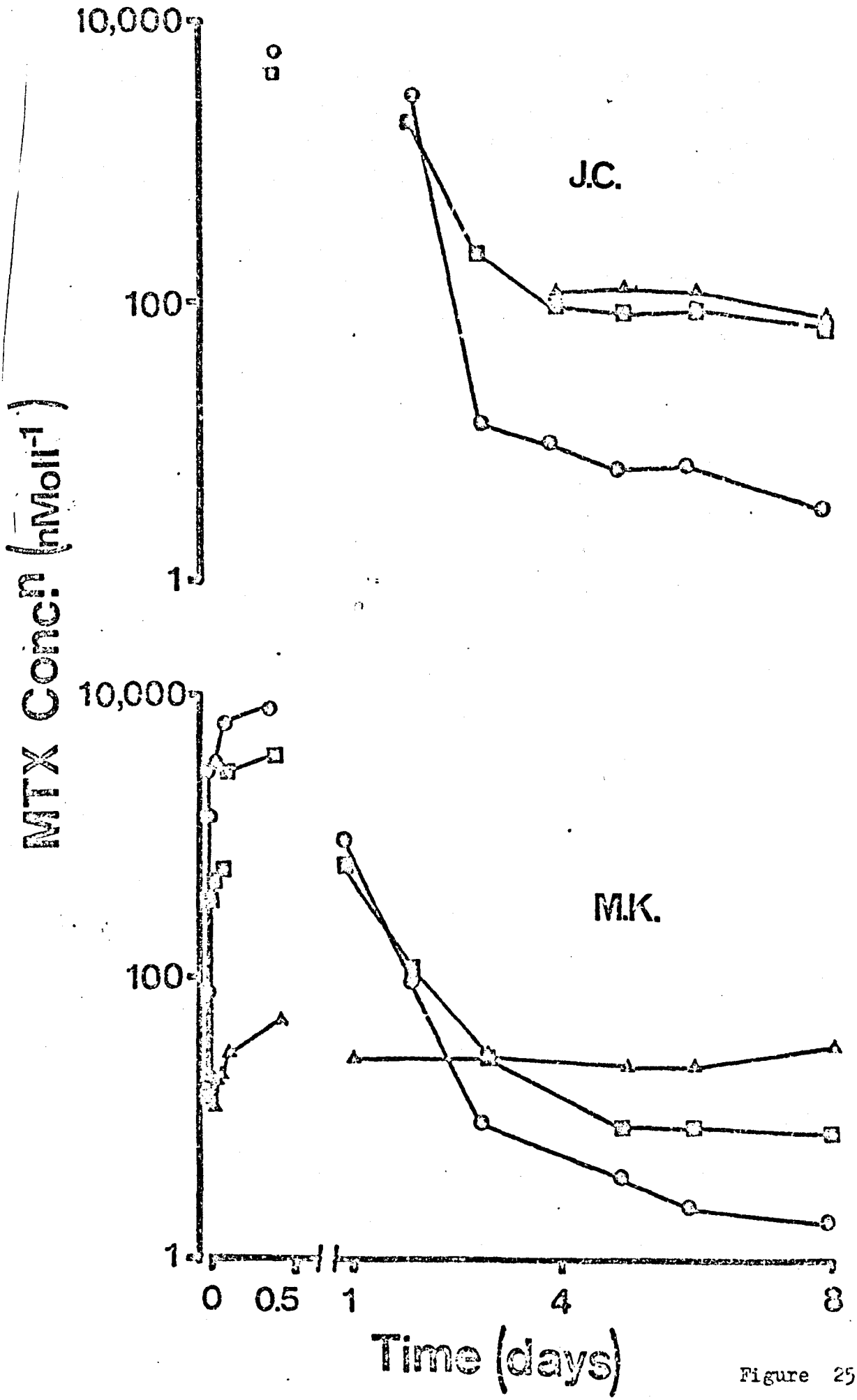


Figure 25

When the study was repeated the concentration in erythrocytes fell to a plateau level and no net increase in drug concentrations was observed at the end of the study after 14 days. The mean drug concentration between 24 h. and 336 h. was 33.58 $mol.l^{-1}$ $\pm$ 3.08 (S.D.). Further, the peak erythrocyte concentration was very much lower, being about 50% of that observed on the first occasion. The peak concentration ratio, whole blood : plasma was also different, being 0.01:1.

Discussion: The persistence of drug in erythrocytes would suggest that MTX is bound in some way either within the cell or on the cell membrane. Indeed it is possible that the process may be described by two mechanisms. The initial rise in concentration during the infusion followed by a sharp drop in erythrocyte concentration immediately following the termination of the infusion would suggest that some MTX is partly associated with erythrocytes in a rapidly reversible manner. This initial association being influenced, perhaps, by the concentration gradient with uptake mainly by diffusion. Also, since, MTX concentration in erythrocytes increased after day 2, in the first treatment of J.M., apparently against a concentration gradient an energy dependent process is possible. This profile is also consistent with erythrocytes being a slowly exchanging kinetic compartment.

However, other explanations for the persistence of MTX in erythrocytes could be postulated. Dihydrofolate reductase activity would be expected to be high in blast cells and may bind considerable quantities of MTX. The increase in erythrocyte MTX levels from day 2 after the first treatment may reflect denovo erythrocyte synthesis and erythrocyte turnover. The MTX levels in the reticuloendothelial system may be considerably higher than serum levels and encapsulation into erythrocytes may occur.

The peak and plateau ratios found in this patient on the first occasion were also considerably different, not only from that obtained when the treatment was repeated but from the other two patients who had received regular chemotherapy. The peak erythrocyte concentration : peak serum concentration was 0.31:1 compared to 0.01:1 found in the other two patients, and when this patient was restudied. Also, when measured on day 8 the plateau erythrocyte level : serum level was 145.50:1 compared to values between 18.75:1 and 34.50:1. It is therefore possible that distribution of MTX between plasma and erythrocytes may change with repeated chemotherapy.

Prolonged exposure to low concentrations of MTX may promote biochemical resistance by mechanisms discussed previously, i.e. associated with decreased binding or altered transport properties. This might explain why the second uptake phase was not observed in the other two patients or when the treatment was repeated in this patient.

The persistence of MTX in erythrocytes may also contribute to toxicity since MTX toxicity is dependent not only on the concentration but also on the length of time exposed to the drug (Goldie, et al., 1972; Young and Chabner, 1973). Therefore even although the concentration in erythrocytes is relatively low, MTX never the less persists for long periods of time after high doses and may exceed the time threshold for toxicity.

Changes in nutritional status could also affect the phenomenon since there is considerable evidence that other folic acid analogues can displace MTX from intracellular sites (Johns et al., 1961; Sheehy et al., 1963; Johns and Penderleith 1963; Johns et al., 1964). However, it would appear that folinic acid did not effect the release of MTX from erythrocytes, since the slope of the concentration/time curve did not alter in response to initiation of 'rescue'. It is possible that the relatively small concentration of folinic acid used in rescue compared to the dose of MTX might not cause detectable displacement of MTX from erythrocytes. Another possibility is that folinic acid is taken up by erythrocytes by the same process and therefore over a similar time course to MTX. Folinic acid may therefore be progressively altering the uptake of MTX, the major effect of which is not observed until later times than covered by this study. In this context it is unfortunate that a pretreatment sample was not withdrawn before the second course of chemotherapy was initiated in patient J.M. Indeed, folinic acid 'rescue' may be, at least partially, responsible for the change in distribution when this patient was studied a second time.

The distribution of MTX between plasma and erythrocytes appears to be mediated by complex mechanisms. Methotrexate persists for a long time in erythrocytes compared to plasma and may therefore contribute to the expression of toxicity by exceeding the time threshold for toxicity. Exposure to low concentrations of MTX for such long periods of time may also promote tolerance as reflected initially in altered association/uptake properties observed when treatment is repeated. The study does not show evidence that folinic acid can influence the distribution of MTX between erythrocytes and plasma. The possibility remains, however, that folinic acid can influence this phenomenon by a more occult mechanism. It is also expected that the progress of disease may have a bearing on the nature of this distribution.

2.2. ABSORPTION STUDIES

2.2.1 Bioavailability of a Methotrexate Oral Syrup Formulation

Methotrexate has been given by mouth for the maintenance of remission in childhood leukaemia for many years. The drug is provided in tablets of 2.5 mg. which is convenient for such low-dose maintenance chemotherapy schedules. Recently intermittent high-dose MTX therapy with folinic acid rescue has been developed in the treatment of solid tumours and lymphomas (Jaffe et al., 1974; Levitt et al., 1973; Turman et al., 1977; Rosen et al., 1974) with subsequent improved efficacy. Clearly such low-dose formulations are unsuitable for high-dose oral therapy. Therefore in an attempt to save patients consuming up to forty or fifty tablets, or being admitted to hospital for intravenous infusions of MTX, an alternative oral formulation in the form of a syrup has been evolved.

The extent of absorption of MTX in oral syrup form was compared with the same dose administered intravenously.

Patients and Methods:

Six patients who were to receive chemotherapy for a variety of solid tumours were given a dose of MTX of 50 mg. m^{-2} either as the syrup or as an intravenous bolus, one week apart, the order being determined on a random basis. After an overnight fast, a heparinised polyethylene catheter was introduced into the left antecubital vein under local anasthaesia. A pre-treatment blood sample was taken and the patient was instructed to empty the bladder. MTX was then administered either by mouth or intravenously into the free arm. A minimum of eleven 5 ml. blood samples were taken at appropriate time intervals up to eight hours. After centrifugation plasma was removed and frozen at -20°C. Urine samples were collected and the volume measured and aliquots of 20 ml. kept for assay of MTX concentration as previously described using ^{75}Se-labelled derivative. The area under the individual MTX serum concentration time curve (AUC) was computed using the trapezoidal rule and the MTX plasma half life ($t_{\frac{1}{2}}$) was derived from the slope of the regression line (log/linear least squares fitting), drawn through the terminal portion of each concentration time curve. The plasma half life ($t_{\frac{1}{2}}$) estimates were compared according to treatment using the Wilcoxon signed ranks test.

Methotrexate Formulation:

The composition of the MTX syrup was as shown in Table 11. The preparation was found to be stable for periods of up to one month in a variety of storage conditions including clear and brown glass bottles at room temperature and at 4°C.

Results:

The sites of tumour in each patient is shown in Table 12. Patient E.D. was found some weeks later at autopsy to have metastases involving the kidney, and patient A.C. had already had a nephrectomy for hypernephroma but had residual tumour. Patient A.F. became progressively debilitated within a short time of starting the study due to a combination of generalised weakness, dehydration and incurrent infection and went into renal failure and died a fortnight after the last dose of MTX. The MTX syrup was tolerated extremely well by all six patients.

There was a three-fold variation in peak MTX concentration and the time taken to reach these peaks varied from 70 - 150 minutes, after oral MTX (Table 13). The area under the oral concentration time curve also varied considerable with a range of 2.26 to 7.94% dose l^{-1} h. In each case the area under the oral curve was very much less than that under the intravenous curve indicating that the absolute bioavailability was poor, the ratio ranging from 15 - 54%. Renal clearance for the first eight hours were compared with serum creatinine levels, but the data failed to show any obvious relationship between these parameters (Table 14). The renal clearance values (0-8 h.) of MTX in patients E.D., A.C. and A.F. were very much lower than in the other three patients, but these values were in accord with their medical history.

No significant group difference could be demonstrated between the plasma half-lives when compared according to route of administration. It was clear, however, when the results were scrutinised that patients E.D., A.C., and A.F. showed differencies in their plasma half-lives between treatment days. These results were explicable in terms of the progressive nature of the disease in these patients.

Constituent	Quantity
Sodium Bicarbonate	20 g.
Injection Methotrexate Liquid B.P.	80 ml (25 mg ml^{-1})
Syrup B.P.	250 ml.
Chloroform Water B.P.	To final volume of 1.0 l.
Final Concentration	10 mg. Methotrexate/5 ml.

Table 11 The composition of the Methotrexate syrup formulation

Patient	Primary Tumour Site	Age	Sex	Dose (mg)	Remarks
C.G.	Bronchus	44	M	70	No side effects
A.P.	Breast and Colon	62	F	50	No side effects
M.B.	Head and Neck	75	M	80	No side effects
E.D.	Bronchus	62	M	95	Diarrhoea Renal Metastases
A.C.	Kidney	57	M	80	Post Nephrectomy
A.F.	Breast	76	F	70	Renal failure

Table 12 Patient particulars including tumour site and dose of drug.

Patient	Oral Peak Concentration µg ml^{-1}	Oral Peak Concentration % Dose l^{-1}	Time to Oral Peak (h)	AUC (% Dose $l^{-1}h$) Oral 0 - 8 h	AUC (% Dose $l^{-1}h$) iv 0 - 8 h	Ratio of AUC oral :AUC iv
C.G.	0.42	0.60	2.5	2.26	10.62	0.21
A.K.	0.72	1.44	2.0	5.12	9.42	0.54
M.B.	0.54	0.70	1.5	2.22	11.52	0.19
E.D.	0.62	0.60	1.8	2.35	13.18	0.18
A.C.	0.74	0.90	1.2	3.0	19.83	0.15
A.F.	1.25	1.80	1.5	7.94	26.46	0.30
Mean	0.71	1.01	1.75	3.82	15.17	0.26
±S.D.	0.29	0.50	0.46	2.30	6.64	0.15

Table 13 Parameters of methotrexate syrup absorption.

Patient	Serum Creatinine ($mg.\ ml^{-1}$)	MTX Renal Clearance ($ml.\ min^{-1}$)	Half Life ($t_{\frac{1}{2}}$) h.	
			Oral	i.v.
C.G.	77	136	2.4	2.8
A.K.	85	130	3.8	3.9
M.B.	67	126	2.1	2.0
E.D.	137	85	3.3	4.1
A.C.	87	61	2.0	4.1
A.F.	87	25	6.3	7.7
mean			3.31	4.10
±S.D.			1.62	1.95
Wilcoxon Test			N. S.	

Table 14 Serum creatinine and pharmacokinetic parameters of methotrexate elimination.

2.2.2 The Absorption of a Series of Different Oral Methotrexate Doses.

The bioavailability of MTX in doses of 50 mg. m^{-2} formulated as a syrup has been shown to be poor and therefore of doubtful therapuetic usefulness. It has been reported in the literature that the absorption of doses greater than 80 mg. m^{-2} are protracted and incomplete leading to poor bioavailability (Henderson et al., 1965; Wan et al., 1974). In contrast the absorption of doses less than 30 mg. m^{-2} are generally well absorbed (Henderson et al., 1965; Huffman et al., 1973; Wan et al., 1974).

Chungi et al., (1978) have studied the kinetics of gastro-intestinal absorption of MTX from the lumen of rat small intestine and demonstrated that absorption obeys Michaelis - Menten kinetics. The above observations have implicated the presence of a saturable intestinal transport mechanism for MTX in man.

The absorption of a series of MTX doses was studied to determine at what concentration saturation of this mechanism occurs when MTX is administered as a syrup.

Patients and Methods:

Four patients with malignant disease participated in this study (Table 15). Each patient was given either a 25, 50, 75 or 100 mg. single oral dose of MTX, followed at weekly intervals by the other three doses. The order of dosing was determined by a Latin square design. Methotrexate formulation was as previously described (Table 11) and was always administered on an empty stomach after an overnight fast. A pre-treatment serum sample was obtained and after drug administration samples were collected frequently for the first 8 h. and at appropriate intervals thereafter until 50 h. Drug levels were measured by radioimmunoassay using ^{75}Se-label. The AUC was computed by the trapezoidal rule and the serum MTX half life values were determined by the slope of the log-linear regression line drawn through the terminal points of the individual concentration time curves.

Results:

The time to peak serum concentration varied from 1 - 2 h. but higher doses were not consistently associated with longer times (Table 15).

Patient	Clinical Details	Dose (mg)	Peak Concentration $\mu g\ ml^{-1}$	Peak Concentration % Dose l^{-1}	Time to Peak (h)	AUC ($\mu g\ h\ ml^{-1}$)	Ratios of $AUC_{n\ mg} : AUC_{25mg}$	Half life $t_{\frac{1}{2}}$ (h)	$t_{\frac{1}{2}}$ Mean ± S.D.
M.C.	Carcinoma of Ovary	25	0.53	2.12	1.5	2.56	1.00	11.0	
		50	1.00	2.00	2.0	4.35	1.70	10.5	
		75	1.15	1.53	1.5	5.21	2.03	12.0	10.95
		100	1.32	1.32	2.0	10.30	4.02	10.3	± 0.76
A.P.	Carcinoma of Breast	25	0.38	1.52	1.0	1.00	1.00	5.0	
		50	0.40	0.80	1.0	1.59	1.58	5.2	7.325
		75	0.47	0.63	1.5	2.03	2.02	13.5	4.12
		100	0.60	0.60	1.5	3.21	3.21	5.6	
J.S.	Carcinoma of Bronchus	25	0.30	1.20	2.0	1.70	1.00	7.0	
		50	0.44	0.88	2.0	2.62	1.54	7.3	7.45
		75	0.51	0.68	1.0	4.01	2.36	7.9	± 0.39
		100	0.83	0.83	1.5	5.11	3.01	7.6	
M.McB	Carcinoma of Lip	25	0.52	2.08	1.0	1.10	1.00	1.4	
		50	0.52	1.04	1.0	1.11	1.01	1.0	1.33
		75	0.85	1.13	1.0	2.38	2.16	1.0	± 0.43
		100	1.20	1.20	1.5	3.81	3.46	1.9	

Table 15 Pharmacokinetic parameters showing dose dependent methotrexate absorption.

There was wide inter-individual variation in the peak concentration obtained for all four doses, ranging from 0.30 - 0.53 $\mu g\ ml^{-1}$ after 25 mg., 0.40 - 1.00 $\mu g\ ml^{-1}$ after 50 mg., 0.44 - 1.15 $\mu g\ m^{-1}$ after 75 mg. and from 0.60 - 1.32 $\mu g\ ml^{-1}$ after 100 mg. However, in each individual the peak height increased with increasing dose. In patient M.C. the peak height increased from 0.53 $\mu g\ ml^{-1}$ after 25 mg. to 1.32 $\mu g\ ml^{-1}$ after 100 mg., in A.P. it increased from 0.38 $\mu g\ ml^{-1}$ to 0.60 μg ml., in J.S. from 0.30 - 0.83 $\mu g\ ml^{-1}$ and in patient M.McB from 0.52 - 1.20 $\mu g\ ml^{-1}$. When expressed in terms of percent dose per litre (% dose 1^{-1}) there was a consistent trend towards lower values as dose increased. In patient M.C. this dropped from 2.12 - 1.32 % dose 1^{-1}, in A.P. from 1.52 - 0.60, in J.S. from 1.20 - 0.83 % dose 1^{-1} and in M.McB this fell from 2.08 - 1.20 % dose 1^{-1}.

There was also a wide inter-individual variation in AUC obtained after each dose. This ranged from 1.00 - 2.56 μg h. ml^{-1} after 25 mg, 1.11 - 4.35 μg h. ml^{-1} after 50 mg. 2.03 - 5.21 μg h. ml^{-1} after 75 mg. and after 100 mg it ranged from 3.21 - 10.30 μg h. ml^{-1}. The ratio, AUC after each dose : AUC after 25 mg. for each individual increased with increasing dose but did not do so proportionately. In patient M.C. the ratio ranged from 1.00 after 25 mg. to 4.02 after 100 mg., for patient A.P. this ranged from 1.00 - 3.21; J.S. from 1.00 - 3.01, and in M.McB from 1.00 - 3.46.

The individual MTX serum half life ($t_{\frac{1}{2}}$) did not alter greatly with increasing dose, the mean values being 10.95 h. $\pm$ 0.76 (M.C.), 7.33 h. $\pm$ 4.12 (A.P.), 7.45 $\pm$ 0.39 (J.S.) and 1.33 h. $\pm$ 0.43 (M.McB). Patient A.P. became progressively dehydrated over the course of the study when given 75 mg MTX, which probably explains why the serum ($t_{\frac{1}{2}}$) was so long. There was, as can be seen above, a wide inter-individual variation in the values of this parameter.

A summary of the individual parameter values are listed in Table 15.

2.2.3 The Effect of Subdivision of Dose on Methotrexate Absorption.

Low doses of MTX have been reported to be almost completely absorbed from the gastro-intestinal tract whereas higher doses are less well absorbed (Henderson et al., 1965; Wan et al., 1974) and these observations have been confirmed by the studies of MTX absorption presented here. This suggests saturation of a specialised intestinal transport process similar to that observed with riboflavine (Levy and Jusko, 1966). Since MTX is a highly polar molecule (Log P octanol = -1.85; Leo et al., 1971) it is unlikely to be absorbed from the intestine by passive diffusion. Further the close structural similarity of MTX to folate vitamins (Fig. 4) points to the strong likelihood of the folate active transport pathway being responsible for the absorption of the drug.

By administering the drug in divided doses of 25 mg. in a formulation designed to delay gastric emptying, and thereby retarding transit of the drug to the site of intestinal absorption, it might be possible to avoid saturation of the uptake process. Since sugar solutions are known to retard the rate of stomach emptying (Wagner, 1973), formulating MTX as its sodium salt in syrup, the above criteria may be conveniently achieved.

The effects of divided doses were compared to single doses containing the same quantity of MTX, both formulated as a syrup.

Patients and Methods:

Eight patients with various forms of malignant disease (Table 16) for whom MTX was considered appropriate therapy, consented to participate in this study. Methotrexate formulation was as previously described (Table 11). Each patient received 100 mg. MTX either as a single dose or else in the form of four divided doses each of 25 mg. taken at two-hourly intervals. The drug was always administered on an empty stomach, after an overnight fast. After a minimum period of one week, each patient received the alternative treatment schedule. The allocation to initial dose schedules (i.e. single or divided dosage) was on a random basis.

Patient	Clinical Details	Order of Study	AUC (ng h ml^{-1})		Peak levels (ng ml^{-1})		Half life (h)		Ratio of AUC 25mg x 4 : AUC 100mg
			100mg	25mg x 4	100mg	25mg x 4	100mg	25mg x 4	
E.L.	Breast Carcinoma Stage II	25mg x 4/100mg	2,657	7,406	487	1,014	6.49	5.61	2.79
J.G.	Carcinoma of Bronchus	25mg x 4/100mg	1,350	4,739	608	640	1.98	4.52	3.51
E.R.	Carcinoma of Bronchus	25mg x 4/100mg	8,829	10,449	1,235	811	11.03	6.96	1.18
A.G.	Breast Carcinoma Skin/Skull secondaries	100mg/25mg x 4	6,436	5,995	929	657	16.22	12.73	0.96
J.H.	Carcinoma of Bronchus	100mg/25mg x 4	4,890	8,883	787	793	11.21	10.17	1.82
C.N.	Anaplastic Lung Carcinoma	100mg/25mg x 4	3,522	7,054	783	690	8.61	7.90	2.00
T.D.	Breast Carcinoma Stage II	100mg/25mg x 4	7,225	7,732	1,309	1,160	4.47	2.97	1.07
A.G.	Penile Carcinoma -inguinal node involvement	100mg/25mg x 4	5,014	7,737	903	961	3.87	4.87	1.54
	Mean		4,990	7,499	880	841	7.99	6.97	1.86
	±		±	±	±	±	±	±	±
	S.D.		2,469	1,724	283	188	4.71	5.22	0.90
			p 0.025		N.S.		N.S.		

Table 16 Pharmacokinetic parameters of methotrexate absorption after a 100mg. bolus and 25mg. x 4.

A pre-treatment serum sample was withdrawn and after the initiation of therapy serum samples were frequently taken during the first 10 h. then at intervals thereafter until 50 h. The complete work sheet for this study is outlined in appendix 1 .

Drug levels were determined by radioimmunoassay using ^{75}Se-labelled derivative. The area under the individual MTX serum concentration time curve (AUC) was computed using the trapezoidal rule. Methotrexate serum half-life ($t_{\frac{1}{2}}$) was derived from the slope of the regression line (log/linear least squares fitting) through the terminal portion of each serum concentration time curve.

Differences in MTX pharmacokinetic parameters between dosage regimens were tested for significance using the Wilcoxon matched pairs signed ranks test. In order to minimise inter-patient variations the kinetic parameters ($t_{\frac{1}{2}}$, peak MTX level and AUC) obtained in each individual were compared according to the treatment regimen.

Results:

Typical serum concentration time curves generated following the administration of 100 mg. and 25 mg. x 4 to a single patient are shown in Fig. 26 . In this example AUC following 25 mg. x 4 is considerably greater than AUC after 100 mg. and this was true of the patient group as a whole. Thus mean AUC after 25 mg. x 4 was 7,499 $\pm$ 1,724 ng. h. ml.$^{-1}$ compared with 4,990 $\pm$ 2,469 ng. h. ml.$^{-1}$ after 100 mg., this difference being significant at the 2.5% level (Table 16).

Peak serum MTX concentrations were similar after both dose forms, mean values being 880 $\pm$ 283 ng. ml^{-1} after 100 mg. and 841 ($\pm$ 188) ng. ml.$^{-1}$ after 25 mg. x 4. Likewise, MTX serum ($t_{\frac{1}{2}}$) values did not differ significantly between treatment days. After 100 mg. the mean ($t_{\frac{1}{2}}$) was 8.0 ($\pm$ 4.7)h. compared with 7.0 ($\pm$ 5.2)h. after 25 mg. x 4. There was, however, marked inter-individual variation in MTX ($t_{\frac{1}{2}}$) values. Scrutiny of the individual results shows that in three patients (E.R., A.G. and T.D.) the ratios $\frac{\text{AUC after 25 mg. x 4}}{\text{AUC after 100 mg.}}$ were near unity. In these three individuals peak MTX levels following 100 mg. were considerably greater than after 25 mg. x 4 and MTX ($t_{\frac{1}{2}}$) values were longer in association with higher MTX peak levels.

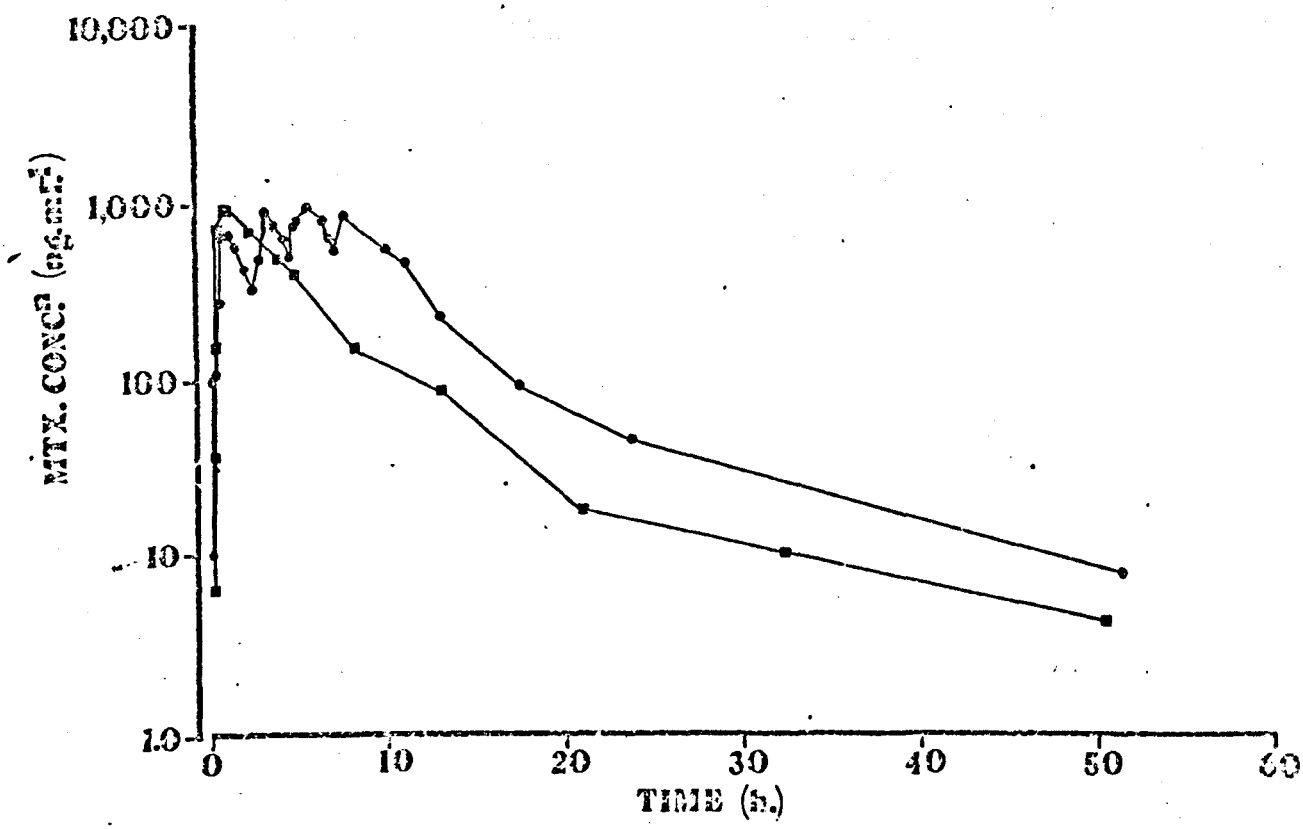


Figure 26. A typical serum concentration/time curve generated following the administration of 100 mg and 25 mg x 4 to patient A.G.

● 25 mg x 4

■ 100 mg

N.B.

The results of this study were presented to the British Pharmacological Society, London, January 1979 and subsequently published.

That dividing high oral doses can improve bioavailability was later confirmed by Christophidis et al., (1979).

Discussion:

The MTX syrup formulation was found to be palateable by the majority of the patients. For the estimation of absolute bioavailability the apparent volume of distribution and the elimination rate constant should be independent of the route of administration. In the determination of the absolute bioavailability of 50 mg. m^{-2}, as in the first study, this is clearly not so. The plasma half lives ($t_{\frac{1}{2}}$) determined for patients E.D., A.C. and A.F. were considerably different between doses (Table 14). It is nevertheless evident that the bioavailability of this dose of MTX in syrup form is extremely poor. The alteration in plasma ($t_{\frac{1}{2}}$) values for MTX in these patients were consistent with the clinical profile in that their disease state was more advanced than was believed at the time of the study. Although kidney involvement per se does not necessarily affect the elimination rate constant, it seems likely that in this case it has, since the 0 - 8 h. MTX renal clearance values were very much lower than in the other three patients.

It was possible to demonstrate that the relative bioavailability of lower doses is better. Doses of 25 mg. were generally much better absorbed than doses of 50, 75 or 100 mg. (Table 15). This observation is in agreement with previously published data (Henderson et al., 1965; Huffman et al., 1973; Wan et al., 1974) which has suggested that doses less than 30 mg. m^{-2} are well absorbed in contrast to doses greater than 80 mg. m^{-2} which are poorly absorbed. It was also evident that there was wide inter-individual variation in the capacity for absorption of MTX in syrup form as indicated by the broad range of values for the area under the MTX concentration time curve, peak concentration and time to peak concentration for the same dose of drug. In patient M.C. (Table 15) the ratio $AUC_{100\ mg.} : AUC_{25\ mg.}$ was 4.02 : 1.0 which was not consistent with the trend set by the other doses. This was observed in spite of a lower peak concentration and a serum ($t_{\frac{1}{2}}$) not much different from the values obtained after the other three doses.

A possible explanation is that the absorption of this dose was somewhat protracted thereby producing a more rounded peak which would increase the value for the AUC. Such a profile was indeed observed for patient M.C. . It was also demonstrated that the AUC is generally greater when the same total dose of the drug was divided into units of 25 mg. given at two hourly intervals. A likely explanation is that by dividing an oral dose overcomes at least partially, saturable intestinal absorption.

Renal clearance, which is the major process of MTX elimination from the body, is by active secretion (Liegler et al., 1969), and it has been reported not to be altered by high doses of drug (Hoffman et al., 1973). The data presented here reveals comparable MTX serum half life values for the two dosage regimens, hence saturation of renal transport processes is unlikely to have been a significant contributing factor to the effect of dividing doses . It is however, noteworthy that three patients (patients E.R., A.G. and T.D., Table 16) with $AUC_{25\ mg.\ x\ 4} : AUC_{100\ mg.}$ ratios near unity all showed considerably higher peak MTX levels and longer serum half lives after 100 mg. than after divided doses. Although it is possible that the intestinal transport systems of these patients had not been saturated by the 100 mg. doses, the data also provides evidence that altered MTX AUC cannot be attributed solely to a bioavailability difference. Further study of MTX renal clearance would seem justified.

The results reported here, however, have immediate relvance to clinical practice. MTX toxicity is known to be dependent on the persistence of drug concentrations above a certain minimum level for a critical time period (Levitt et al., 1973), and this may also be true when considering response to MTX therapy. Increasing AUC after oral MTX would thus contribute to enhancement of MTX effects overall (efficacy and toxicity) so that an improved response to chemotherapy will result if toxicity can be satisfactorily controlled (e.g. using citrovorum factor). Dividing therapeutic doses into units of 25 mg. given two hourly may therefore permit oral administration to be considered as a reasonable alternative to intravenous therapy.

2.3. PROTEIN BINDING STUDIES

2.3.1. Validation of Methods

The main limitations of continuous ultrafiltration, as indeed for simple ultrafiltration, are:-

1) The requirement for a suitable membrane; and

2) The accuracy and specificity of the drug assay procedure.

Most drugs are bound or retained on ultrafiltration membranes to some extent although these are made from inert material. In order to test the suitability of membranes, drug recoveries where measured when only buffer was present in the ultrafiltration chamber. However, the assessment of the membranes proved to be far from simple. When the concentration of drug in the ultrafiltrate (Df) was plotted against the concentration in the M.M.C. chamber (Dc) a series of lines were generated (Fig.27) which depended on the flow rate which in turn was related to the gas pressure at which the experiment was run. The observed membrane effect was therefore the sum of the membrane binding and a practical experimental factor which depended on the apparatus. The overall effect was measured by defining a reflection coefficient (σ) which related the drug concentration in the ultrafiltrate to the concentration in the M.M.C. chamber:-

$$\sigma = 1 - \frac{Df}{Dc}$$

This value was measured at the flow rate encountered during protein binding experiments, i.e. 3 ml h^{-1}. This process was also used to determine the relative suitability of continuous ultrafiltration and simple ultrafiltration. A comparison of these techniques for methotrexate, salicylate, sulphadiazine, phenylbutazone, diphenylhydantoin, cyclophosphamide and adriamycin using XM_{50}, PM_{10} and UM_{10} Diaflo untrafiltration membranes was made (Table 17) to validate the systems for the measurement of drug-protein binding interactions.

Considerable advantage over simple ultrafiltration was obtained by employing continuous ultrafiltration for each of these compounds, since the reflection coefficient was much lower by this method.

REFLECTION COEFFICIENT (σ)*

Drug	Continuous Ultrafiltration			Simple Ultrafiltration		
	XM_{50}	PM_{10}	UM_{10}	XM_{50}	PM_{10}	UM_{10}
Methotrexate	0.01	0.10	-	0.20	0.35	-
Salicylate	0.05	0.06	0.13	0.25	0.35	0.51
Sulphadiazine	0.10	0.10	0.10	0.54	0.81	0.29
Phenylbutazone	0.27	0.35	-	0.35	0.47	-
Diphenylhydantoin	0.07	0.14	0.09	0.77	0.50	-
Cyclophosphamide	0.01	0.01	-	-	-	-
Adriamycin	0.05	0.10	-	-	-	-

* Reflection Coefficient $\sigma = 1 - \frac{Df}{Dr}$

Table 17 The reflection coefficient of a number of drugs obtained by simple and continuous ultrafiltration

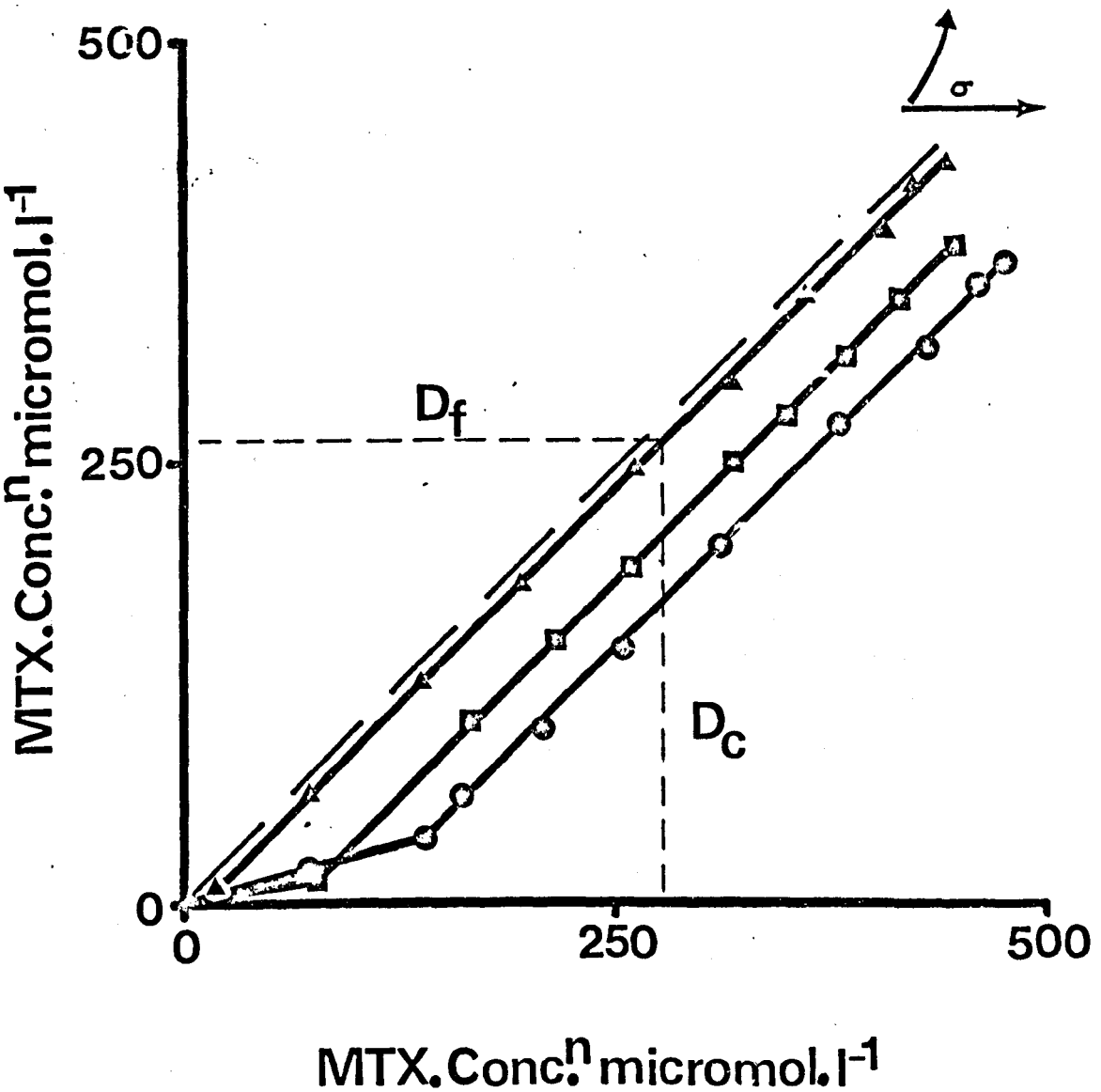


Figure 27 The influence of flow rate on drug recoveries from the MMC chamber

Further, when employing simple ultrafiltration, considerable differences between membrane batches was observed, which could alter drug recoveries by as much as ± 15%. Simple ultrafiltration is therefore not a wholly satisfactory method for the estimation of protein binding parameters. In contrast continuous ultrafiltration can be employed with confidence to the assessment of drug-protein binding phenomena. Since the reflection coefficient for methotrexate using XM_{50} membranes was consistently very low (0.01) no correction factor for membrane effect was considered necessary.

The accuracy, which embraces the concepts of both precision and sensitivity, and specificity of the radioimmunoassay method has been discussed. It was concluded that this assay method was an acceptable procedure for the measurement of drug concentrations in the elucidation of binding parameters by continuous ultrafiltration. However, large cumulative errors could potentially occur, since the chamber concentration is the sum of a number of preceding estimates. Therefore, at the end of each experiment, the calculated total concentration was compared to the value obtained by assay of the concentration of the drug in the chamber. The experimental results were rejected if the calculated concentration differed by more than 15% from the assayed value.

2.3.2. Binding of Methotrexate to Different Serum Protein Components

Normal human serum contains a total complement of approximately 70 g l^{-1} of protein. Serum protein, of which there are over twenty-five distinct entities, differ in size, shape, composition, charge, solubility and chemical reactivity (Goldstein, 1949). There are a number of these serum proteins which are capable of binding or interacting with a large number of exogenous and endogenous compounds. Before examining the nature of the methotrexate-protein interaction it was necessary to determine to what serum proteins this agent will bind.

Method A 10 µl aliquot of ^{3}H - MTX was added to 200 µl of human serum pooled from eight normal subjects, mixed thoroughly and placed in an incubator at 37°C. for 30 minutes to ensure equilibrium had been reached. The serum proteins in 20 µl of this mixture were then separated by electrophoresis on cellulose acetate strips at pH 8.6 (barbiturate buffer I = 0.05). A narrow band along the side of the cellulose acetate strip was cut off and stained by 0.2% ponceau S. in 3% trichloracetic acid and washed in 5% acetic acid. The resulting bands were used to locate the various proteins on the electrophoresis strip (Fig. 28). The protein bands were cut off and eluted with 0.5 ml. of 0.01 N sodium hydroxide and the radioactivity in 100 µl counted on a Hewlett Packard Tricarb liquid scintilation counter (Table 18). Quenching, determined by the channels ratio method, was low (12%).

Results Methotrexate is bound to serum proteins predominantly to serum albumin, 87.3% of activity being present in this protein fraction.

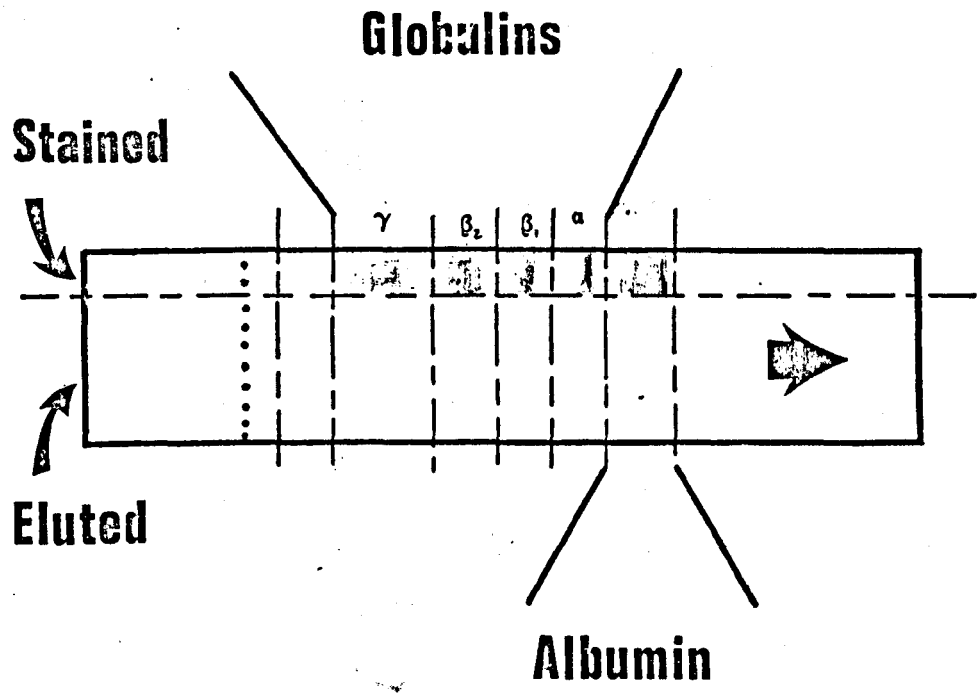


Figure 28 The separation of serum proteins on cellulose acetate strips

Serum Protein	Counts/min.	% Bound
Albumin	2688	87.30
Globulins		
α	108	3.51
β_1	107	3.48
β_2	115	3.73
γ	61	1.98

Table 18 Percentage of MTX bound to the different protein components of serum.

2.3.3. The Protein Binding of Methotrexate by the Serum of Normal Subjects

The protein binding studies of MTX reported in the literature have been of a limited nature and have yielded disparate results (Henderson, et al., 1965; Liegler et al., 1969; Kock-Wesser and Sellers, 1976; Taylor and Halprin, 1977). It was therefore necessary to examine the interaction between MTX and albumin in normal human serum.

Since MTX has found application in the treatment of psoriasis and in long-term treatment of breast carcinoma, in which conditions the patients are relatively fit, the results may have immediate clinical relevance.

Subjects Only serum from subjects with no clinical or biochemical manifestation of disease was used (Table 19).

Methods The protein-methotrexate interaction was elucidated by continuous ultrafiltration using the Amicon Diaflo XM_{50} membranes as previously described (Page 67). A reservoir concentration of 20-25 x 10^{-6}M was employed which enabled the study of the protein binding over a range in total concentrations of 5-50 x 10^{-6}M. For the determination of binding parameters a reservoir concentration of 750 x 10^{-6}M was used and the binding examined over a total concentration range of 50-1000 x 10^{-6}M. This range in concentrations was necessary for the construction of satisfactory Scatchard plots. The exact reservoir concentration in different experiments was determined by assay of an aliquot of the solution, and whichever experimental condition was employed the bound and unbound drug in 12-16 samples was determined.

The serum albumin concentration was measured using the bromocresol green method by a technicion Auto-Analyser. Methotrexate concentrations were measured by radioimmunoassay using the ^{75}Se-MTX label. Curve fitting for the Scatchard plots was carried out on the Northumbrian Universities Multiple Access Computer (NUMAC) using a least quares fitting programme based on Numerical Analysis Group (NAG) routines.

Subject	Sex	Age (Y)	Ast (iul^{-1})	Alt (iul^{-1})	Serum Bilirubin $(\mu moll^{-1})$	Serum Albumin (gl^{-1})	Class I N1	Class I K1 x $10^4 M^{-1}$	Class II N2	Class II K2 x $10^4 M^{-1}$
M M^{c}A	F	35	22	17	8	41	0.25	19.4	1.58	0.09
D.G.	M	18	20	21	9	43	0.18	43.6	1.92	0.10
E.B.	F	22	28	15	10	49	0.13	95.1	3.52	0.03
E.R.	F	35	19	12	5	42	0.17	31.6	1.05	0.24
F.B.	F	33	21	18	13	45	0.18	124.0	2.89	0.07
W.S.	M	31	19	23	12	44	0.12	91.6	2.74	0.20
C.M^{c}N	M	25	26	22	6	46	0.09	77.4	1.38	0.21
S.M.	M	30	26	18	7	41	0.12	86.5	1.03	0.49
Mean		28.6	22.60	18.25	8.75	43.88	0.16	71.15	2.01	0.18
±S.D.		6.30	3.54	3.69	2.82	2.75	0.05	35.98	0.93	0.15

Table 19 Biochemical values and binding parameters in eight volunteers.

Results Linear protein binding of 94.24% ± 3.67 was observed until the total concentration exceeded 30 x 10^{-6}M. Between 30-50 x 10^{-6} Mol l^{-1} small alterations of only 2-4% occurred. No great change in the percentage bound therefore occurred until the concentration exceeded 50 x 10^{-6} Mol l^{-1}, when it rapidly decreased (Fig. 29). Examination of Scatchard plots indicated two distinct groups of binding sites. In the high affinity group (Class I) there were 0.16 ± 0.05 (S.D.) binding sites (N_1) with an intrinsic association constant (K_1) of 71.15 x 10^4 M^{-1} ± 35.98 (S.D.), whereas in the lower affinity group (Class II) N_2 = 2.01 ± 0.93 binding sites and the association constant K_2 = 0.18 x 10^4 M^{-1} ± 0.15 (Table 19).

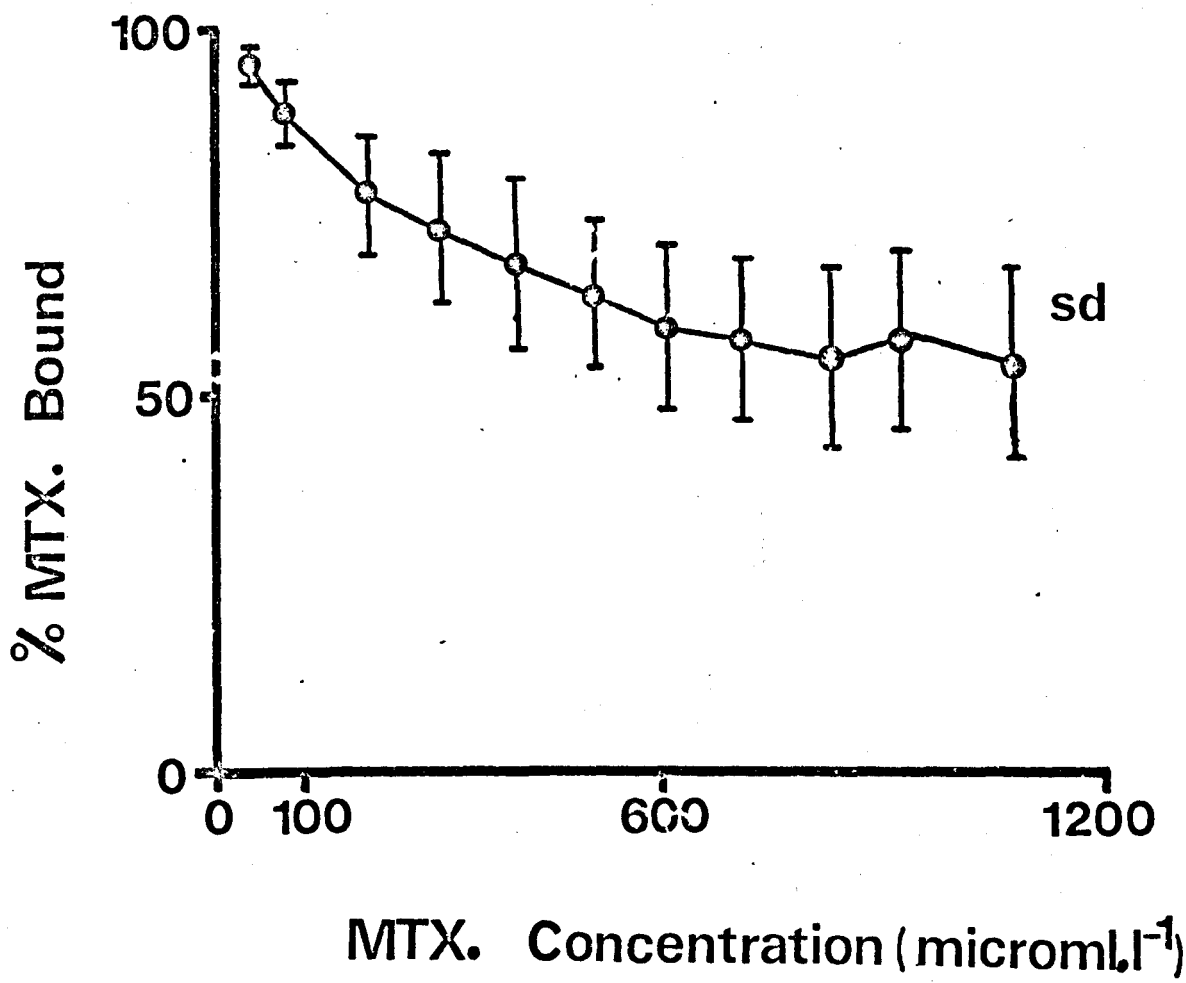


Figure 29 The effect of increasing serum methotrexate concentrations on the percent bound drug

2.3.4. The Protein Binding of Methotrexate by the Serum of Patients with Neoplastic Disease.

To approach maximum inhibition of DNA synthesis enough MTX must be present intracellularly in concentrations greater than necessary to saturate dihydrofolate reductase (Goldman, 1974). It is generally accepted that plasma protein binding can influence the amount of drugs entering cells (Van Os, 1964; Martin, 1965; Booker and Darcey, 1973; Schoeman and Azarnoff, 1975; Levy, 1976; Wagner, 1976; Gibaldi and McNamara, 1978). Further, albumin metabolism (Steinfeld, 1960; Waldman, et al., 1962) and electrophoretic behaviour (Petermann et al., 1948; Rotfino, et al., 1948; Neely and Neil, 1956) can be considerably modified by the presence of neoplastic disease. Alterations in the nature of albumin-methotrexate interaction in neoplastic disease is therefore possible but this has not been explored. Knowledge of an individuals protein-MTX binding and anomalies therein may therefore be crucial in establishing effective chemotherapy with minimum toxicity.

Patients: Recently diagnosed patients admitted to hospital consented to donate 30 ml. of blood before initiation of chemotherapy for a variety of neoplastic disease states. Table 20 . gives patient particulars and some relevant biochemical values. Patient data was compared to results obtained from an age and sex matched 'control' group of patients attending an outpatients clinic for a number of non-malignant conditions (Table 21).

Methods: The methods employed for the elucidation of binding parameters have been described previously (Page 67). A reservoir concentration of 750 x 10^{-6}M was used as before which allowed the study of protein binding over the total concentration range of 50-1000 x 10^{-6}M necessary for the construction of Scatchard plots. Group comparisons between normal subjects and patients were made by the Mann Whitney 'U' test.

Patient	Sex	Age (Y)	Disease	AST (iul^{-1})	ALT (iul^{-1})	Serum Bilirubin ($\mu mol\ l^{-1}$)	Serum Albumin (gl^{-1})
E.G.	F	66	Carcinoma Breast	23	11	5	45
E.L.	M	63	Carcinoma Lung	-	-	7	44
R.S.	M	48	Brain Tumour	17	30	14	39
J.S.	M	40	Carcinoma Lung	19	19	6	38
E.R.	F	62	Carcinoma Breast	22	12	6	40
A.P.	F	49	Carcinoma Breast	24	17	6	46
D.P.	M	53	Osteo-sarcoma	13	18	6	41
H.B.	F	73	Carcinoma Caecum	21	18	7	32
M McB	M	63	Carcinoma Lip	18	13	4	36
M.D.	F	71	Melanoma	22	13	6	38
C McS	F	80	Lymphoma Colon	19	16	65	37
A.T.	F	64	Carcinoma Lung	20	11	6	35
C.T.	F	77	Carcinoma Breast	20	16	4	29
M.C.	F	56	Carcinoma Ovary	58	42	9	43
Mean		61.79		22.77	18.62	10.79	38.79
±S.D.		11.56		10.96	8.59	15.80	4.89

Table 20 Clinical details and biochemical valves of the patient group.

Controls	Sex	Age (Y)	Disease	AST (iul^{-1})	ALT (iul^{-1})	Serum Bilirubin ($\mu mol\ l^{-1}$)	Serum Albumin (gl^{-1})
M.R.	F	67	Diabetic Hypertensive	14	17	11	39
E.B.	F	79	C.V.A.	21	18	7	32
E.C.	M	70	C.V.A.	23	24	6	36
R.J.	M	45	C.V.A.	32	20	15	37
M.B.	F	64	C.V.A.	18	17	7	40
W.A.	F	56	Coeliac	-	-	11	28
J.A.	F	56	Coeliac	22	17	29	34
J.P.	M	76	Angina	16	11	11	35
Mean		64.13		20.86	17.71	12.13	35.13
±S.D.		11.35		5.90	3.90	7.43	3.87

Table 21 Clinical details and biochemical values of the control group.

Curve fitting for the Scatchard plots was carried out on the Northumbrian Universities Multiple Access Computer (NUMAC) or on the Varian V71 computer using a least squares fitting programme. The fitting was found to be satisfactory by either system and comparison of results obtained by fitting on both computers showed almost identical results (differences occasionally occurring only at the second decimal place in parameter values).

Results: No significant difference could be demonstrated between the percent bound in the control group and the patients with neoplastic disease (Table 22). The mean protein binding at a total concentration of 50×10^{-6}M was 92·04 ± 4·04 (S.D.) in the controls compared to 92.77% ± 4.50 (S.D.) in the patients.

Few biochemical abnormalities were observed but a low serum albumin was found in one patient (C.T., 29 gl^{-1}) and one control (W.A., 28 gl^{-1}). The percent bound in both subjects was considerably reduced, being 81.10% and 82.97% bound respectively. But the nature of the methotrexate - protein interaction did not alter in response to hypoalbuminaemia in these subjects, since the binding parameters did not differ greatly from the mean value in their respective groups (Table 23;24). A high serum bilirubin was observed in patient C.McS (65×10^{-6}M) which again did not appear to affect the nature of the drug-protein interaction. The percentage bound MTX may, however, be altered although the reduction in binding is small, being 88.85% compared to the group mean of 92.77% ± 4.50 (S.D.).

Inspection of the Scatchard plots generated by both groups suggested two classes of binding sites. The analysis of these curves in the control group indicated 0.19 ± 0.04 (S.D.) binding sites (N_1) with an intrinsic association constant (K_1) of 45.04×10^4 M^{-1} ± 7.34 (S.D.) in the high affinity group (Class I). In the low affinity group (Class II) N_2 = 2.31 ± 1.53 (S.D.) and $K_2 = 0.16 \times 10^4$ M^{-1} ± 0.10 (S.D.) (Table 22). In the group of patients with malignant disease N_1 = 0.19 ± 0.04 (S.D.). $K_1 = 51{\cdot}29 \times 10^4$ M^{-1} ± 30.10 (S.D.) and N_2 = 3.06 ± 1.79 (S.D.). $K_2 = 0.11 \times 10^4$ M^{-1} ± 0.10 (S.D.) (Table 22).

Group	Age (Y)	Serum Albumin (gl^{-1})	% Bound*	Class I		Class II	
				N_1	$K_1 \times 10^4\ M^{-1}$	N_2	$K_2 \times 10^4\ M^{-1}$
Patients	61.79 ± 11.56	38.79 ± 4.89	92.77 ± 4.50	0.19 ± 0.04	51.29 ± 30.10	3.06 ± 1.79	0.11 ± 0.10
Controls	64.13 ± 11.35	35.13 ± 3.87	92.04 ± 4.04	0.19 ± 0.04	45.04 ± 7.34	2.31 ± 1.53	0.16 ± 0.10
Mann Whitney 'U' Test	N.S.	N.S.	N.S.	N.S.	--	N.S.	N.S.

Table 22 Group comparison of patient and control groups.
Values refer to binding at a total drug concentration of 50 μM.

Controls	% Bound*	Class I N_1	Class I $K_1 \times 10^4\ M^{-1}$	Class II N_2	Class II $K_2 \times 10^4\ M^{-1}$
M.R.	93.90	0.16	44.72	0.55	0.31
E.B.	92.01	0.18	55.95	5.04	0.04
E.C.	93.60	0.23	38.91	4.01	0.09
R.J.	93.58	0.14	32.88	1.44	0.21
M.B.	96.02	0.21	49.63	1.93	0.13
W.A.	82.97	0.27	43.78	1.70	0.15
J.A.	90.16	0.18	42.73	2.72	0.06
J.P	94.11	0.15	51.73	1.06	0.25
Mean	92.04	0.19	45.04	2.31	0.16
±S.D.	4.04	0.04	7.34	1.53	0.10

Table 23 Individual protein binding data of the control group. Values refer to binding at a total drug concentration of 50 μM.

Patient	% Bound*	Class I N_1	Class I $K_1 \times 10^4\ M^{-1}$	Class II N_2	Class II $K_2 \times 10^4\ M^{-1}$
E.G.	92.99	0.26	12.20	4.13	0.03
E.L.	91.73	0.23	14.70	5.56	0.03
R.S.	98.04	0.17	133.00	1.86	0.12
J.S.	97.64	0.20	90.20	3.11	0.05
E.R.	96.13	0.17	50.00	5.73	0.05
A.P.	93.30	0.18	48.00	4.02	0.05
D.P.	90.75	0.17	51.18	5.52	0.05
H.B.	92.77	0.21	35.07	3.96	0.04
M McB	90.06	0.13	56.76	1.19	0.38
M.D.	91.50	0.13	51.35	1.39	0.23
C McS	88.85	0.16	36.00	1.04	0.20
A.T.	96.83	0.24	50.18	0.70	0.06
C.T.	81.10	0.22	37.87	2.86	0.05
M.C.	97.09	0.18	51.50	1.75	0.15
Mean	92.77	0.19	51.29	3.06	0.11
±S.D.	4.50	0.04	30.10	1.79	0.10

Table 24 Individual protein binding data of the patient group. Values refer to binding at a total drug concentration of 50 μM.

No significant difference could be demonstrated when the groups were compared (Table 22). There was a greater scatter of values around the mean for K_1 in the patient group (Table 22) due mainly to the very high values obtained in patients R.S. and J.S. There was no biochemical or obvious clinical abnormality to which these anomalous values could be related.

Discussion: A large number of treatment schedules using MTX have been developed, such as high dose therapy with folinic acid rescue (Levitt, et al., 1973; Pratt, et al., 1975; Stoller, et al., 1977) in an endeavour to gain the greatest therapeutic response with tolerable toxicity. It is surprising that this approach has not included studies of the nature of MTX - serum protein interaction. Protein binding could be of critical importance in that anomalies therin might greatly affect the distribution and excretion of this agent. Further, the studies of MTX protein binding which have been recorded have suffered from certain irreconcilable deficiencies. Simple ultrafiltration was used in some studies to separate 'bound' and 'free' drug (Liegler, et al., 1969; Taylor and Halprin, 1977) so non-specific binding to membranes or ultracentrifugation cones cannot be eliminated. The MTX concentrations were not assayed but rather radio-labelled drug was employed to estimate the percentage bound (Henderson, et al., 1965; Taylor and Halprin, 1977; Liegler, et al., 1969). Crystalline serum albumin reconstituted in phosphate buffer has also been used (Taylor and Halprin, 1977) and this cannot be considered to be physiological in nature. These deficiencies probably explain the discrepancies between published studies (50-94%) and the results of percentage binding reported here (94.24 ± 3.67%). In the present study the MTX concentrations in the ultrafiltrate were measured by a specific radioimmunoassay of high precision. Further, continuous ultrafiltration overcame many of the membrane binding problems and individual sera were used rather than reconstituted crystalline albumin. The system described here is thus an invitro system which more closely reflects that occurring in vivo.

It is widely accepted that the protein binding of a drug can modify its distribution in the body (Van Os, 1964; Martin, 1965; Levy, 1976; Wagner, 1976; Gibaldi and McNamara, 1978) and in so doing also influence the dose response relationships including toxicity as only the free drug concentration contributes to the rate of transport across cell membranes.

Further, the amount of drug which a protein can bind depends on the total drug concentration, the protein concentration, the number of binding sites on the protein and the association constant.

According to Van Os (1964) plasma proteins will only have an appreciable effect on drug distribution if the drug has an intrinsic association constant greater than $1 \times 10^4\ M^{-1}$. He also predicted that drugs which bind with a high affinity will have a dosage-range within which small increases in dose will produce relatively large increases in the concentration of free drug. This appears to be true of MTX since its high affinity sites have an intrinsic association constant greater than $1 \times 10^4\ M^{-1}$ and increasing a total concentration of 50 μMol. l^{-1} by 1.5 times will increase the free drug concentration by 2-3 times in the majority of patients. MTX therefore shows the non-linear protein binding phenomenon predicted for drugs with a high association constant. Such total serum concentrations (50 μMol. l^{-1}) however, are attained only under a high dosing regimen. It is likely that the improvement in the therapeutic index obtained by high dose MTX chemotherapy is at least partially related to the greater increase in free drug rather than on the increase in total drug concentration.

It is also likely that the percent bound MTX will be dependent on the concentration of serum albumin, particularly as albumin is the main protein component in serum to which MTX binds. However, the binding of MTX remains reasonably steady over a wide range in concentrations, (1-50 μMol. l^{-1}). It is possible that the capacity of serum albumin even in reduced concentrations, to bind MTX would be sufficient at the low serum levels of drug. Conversely, high dosing regimens may result in a pronounced increase in available drug. In the two subjects with hypoalbuminaemia, assuming the same degree of binding in vivo, two - three times more MTX would be available at a concentration of 50 μMol. l^{-1} than in patients with normal serum albumin levels.

The effect of this situation in terms of response (efficacy and toxicity) is difficult to predict at present. It could potentially be a major factor in deciding, not only the dose of MTX, but also the extent of folinic acid 'rescue' given to individual patients.

The presence of neoplastic disease did not appear to modify the interaction of MTX with serum albumin when the parameter values were compared to the controls. As patients were only recently diagnosed and were otherwise reasonably fit it is perhaps not surprising that the results did not show wide deviations from controls. Neoplastic disease can nevertheless result in cachexia in which alterations in intermediary metabolism may in turn affect the nature of MTX protein binding due to aberrent albumin metabolism. In addition, prolonged treatment with MTX might be associated with alterations in the binding properties of the drug depending on changing nutritional status associated with response (or failure of response) to therapy. The concurrent use of other drugs (cytotoxics, analgesics, antiemetics, antibiotics etc.) may complicate protein binding by competing for binding sites or displacing MTX from sites which it already occupies. The results reported here are only the first part of a study to determine the effect on MTX protein binding, if any, of these factors. It is, however, interesting that there was a wide scatter of values for K_1 in the patient group which may be the start of a progressive binding aberration in some patients depending on the factors mentioned above.

2.4 ELIMINATION STUDIES

2.4.1. Renal Clearance of Methotrexate

Methotrexate is used, in clinical practice, over a wide dose range. In the light of this there is surprising paucity of published data relating the pharmacokinetics of the drug to dose range studied. Some evidence favouring saturable methotrexate elimination has accumulated but the range of plasma levels at which non-linear pharmacokinetics may apply is unclear.

A disproportionate increase in plasma methotrexate concentration followed infusion of a 20 g. dose of the drug (Goldie, et al., 1972) but another group of workers reported roughly proportional peak serum methotrexate levels with dose over a 50 to 200 mg. Kg^{-1} range (Stoller, et al., 1975).

Attempts to model the pharmacokinetics of high dose methotrexate infusion led to postulation of a saturable renal elimination process (Leme, et al., 1975; Reich, et al., 1977) though the extent to which this is quantitatively important is unclear. Recently, however, Shen and Azarnoff (1978) have reported preliminary findings consistent with a marked fall-off in methotrexate renal clearance at plasma concentration levels above 0.1 $\mu g\ ml^{-1}$.

The impetus to study this particular aspect of the pharmacokinetics of methotrexate stems in fact not from the suggestions in the literature but from a previous study (see page 99 of this thesis) primarily designed to assess bioavailability of the drug after divided doses (Calman, et al., 1979). In this study three out of eight patients showed prolongation of methotrexate's apparent terminal half-life in association with higher peak levels of the drug - an effect consistent with saturable elimination. Accordingly the present study was designed to examine specifically the effect of dose on the clinical pharmacokinetics of methotrexate.

Methods:

Six patients receiving methotrexate for the treatment of a variety of malignant diseases consented to take part in the study. The clinical details of these patients are summarised in Table 25. All but one received combination chemotherapy as indicated but, for the purpose of this study administration of other drugs was delayed until 24 h. after MTX dosing. Following cannulation of veins in both antecubital fossae, patients received a bolus intravenous MTX injection - 25 mg. followed after a minimum of one week by 100 mg. (or vice versa - the order of administration behind random). Samples for MTX serum determination were removed at appropriate times until 48 h. after drug administration. Urine for MTX assay was also collected up to 48 h. after drug administration.

Methotrexate concentrations in serum and urine were measured by radioimmunoassay using the ^{75}Se-labelled derivative.

Areas under the MTX serum concentration-time curves were estimated using the logarithmic trapezoidal rule. The logarithmic rather than the linear trapezaidal rule was used in view of the long intervals between sampling times after 12 h. this is in keeping with the recommendation of Chiou (1978). Terminal MTX serum half lives were derived from log-linear least squares fitting of the final portion of the concentration-time curves and these were used to derive $AUC_{0-\infty}$. Renal clearances were calculated until 48 h. only because of the difficulty in extrapulating urinary drug recovery to infinity.

Within-subject differences in these parameters were tested for significance using the Wilcoxon matched-pairs signed-rank test.

Results:

Fig. 30 shows the MTX serum concentration-time curves for all six patients and Table 26 summarises the MTX levels attained followed bolus intravenous injection of the drug. Mean MTX serum-concentration-time curves after 25 mg. and 100 mg. are also shown for the six individuals (Fig. 31).

Patient	Diagnosis	Additional Therapy	Serum Creatining (μmol l^{-1})
A M^{c}G	Carcinoma of Breast	Cyclophosphamide 1g 5-Fluorouracil 500 mg	89
W.T.	Carcinoma of Breast	Cyclophosphamide 1g 5-Fluorouracil 500 mg	97
M.T.	Ovarian Carcinoma	Cyclophosphamide 1g 5-Fluorouracil 500 mg	90
M M^{c}I	Carcinoma of Breast	Cyclophosphamide 1g 5-Fluorouracil 500 mg	68
V.L.	Carcinoma of Breast	Cyclophosphamide 1g 5-Fluorouracil 500 mg	52
J.G.	Penile Carcinoma	None	-

Table 25 Clinical details of patients.

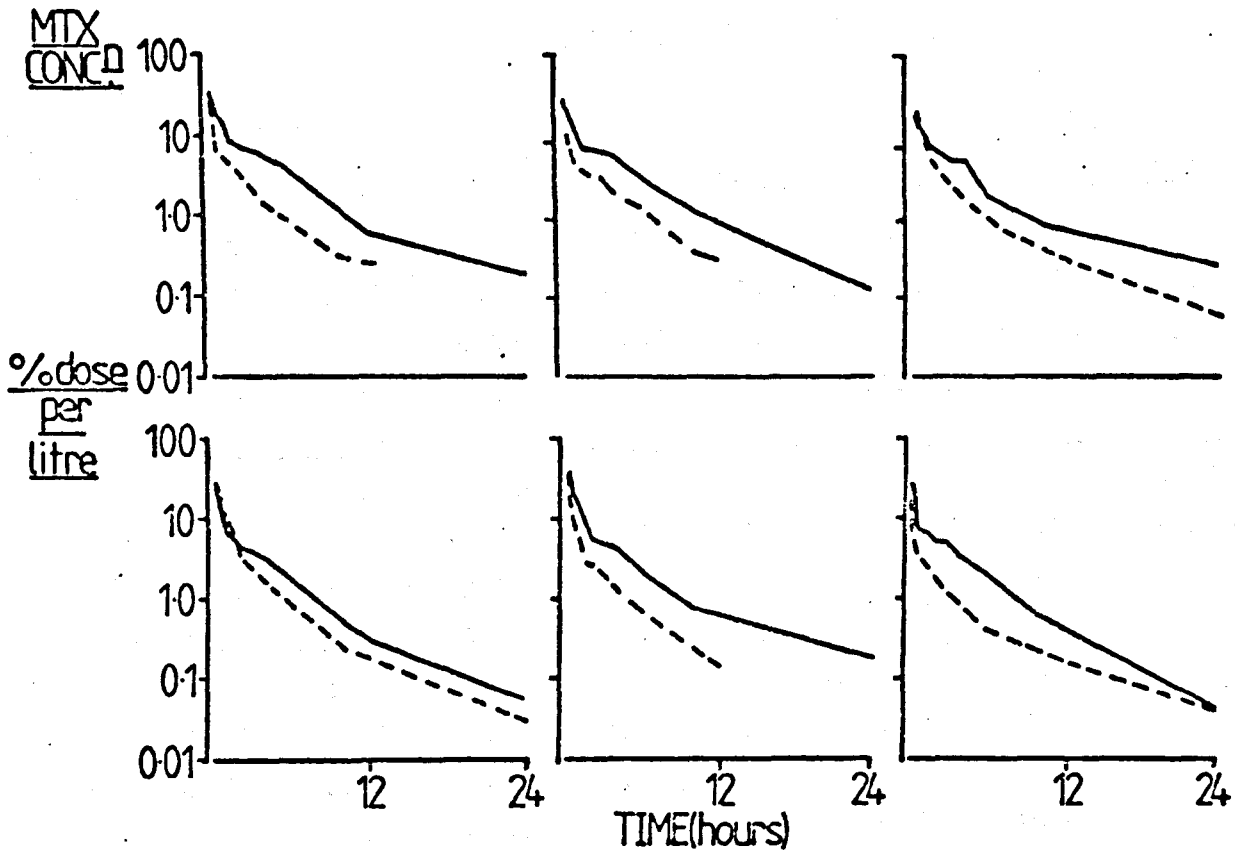


Figure 30 Concentration/time curves of each patient after 100mg. and 25mg. i.v. bolus injection.

— — — 25mg

——— 100mg

Concentration (% Dose l^{-1})

Time	Patient	A.McG.		W.T.		M.T.		M.McI.		V.L.		J. .	
	Dose (mg)	25	100	25	100	25	100	25	100	25	100	25	100
10 min.		24.46	31.00	25.43	29.32	-	-	28.00	24.60	18.40	36.53	16.56	27.82
20 min.		16.52	23.05	12.42	22.05	21.70	18.00	21.60	15.60	13.60	21.02	5.32	18.59
40 min.		6.38	17.02	6.79	16.32	15.15	14.00	12.80	11.20	7.20	10.69	3.64	7.58
1 hour		5.44	14.16	4.56	12.43	10.34	11.60	10.96	6.73	5.53	8.13	3.00	6.59
1.5 hours		4.48	8.74	3.80	7.54	5.04	7.61	5.22	5.34	2.82	8.05	2.40	6.35
2 hours		3.60	7.36	3.40	7.10	4.08	6.51	3.57	4.38	2.61	5.03	1.94	5.22
3 hours		2.40	6.45	3.12	6.40	2.76	5.37	-	3.88	1.80	4.60	1.30	5.11
4 hours		1.52	5.59	1.92	5.60	1.88	5.02	1.60	3.00	1.24	4.04	0.85	3.34
6 hours		0.88	3.88	1.32	2.95	1.01	1.92	-	-	0.66	2.08	0.42	2.02
10 hours		0.32	1.12	0.37	1.20	0.68	0.88	0.25	0.54	0.24	0.77	0.22	0.62
12 hours		0.27	0.61	0.29	0.86	0.30	0.70	0.18	0.30	0.15	0.62	0.16	0.42
24 hours		U.D.	0.19	U.D.	0.12	0.06	0.25	0.03	0.27	0.01	0.19	0.04	0.04
36 hours		U.D.	0.07	U.D.	0.02	U.D.	0.13	U.D.	0.01	U.D.	0.06	U.D.	0.01
48 hours		U.D.	0.02	U.D.	0.01	U.D.	0.06	U.D.	U.D.	U.D.	0.02	U.D.	U.D.

U.D. = Undetectable.

Table 26 Individual data points for each patient obtained after 100mg. and 25mg. i.v. bolus injection.

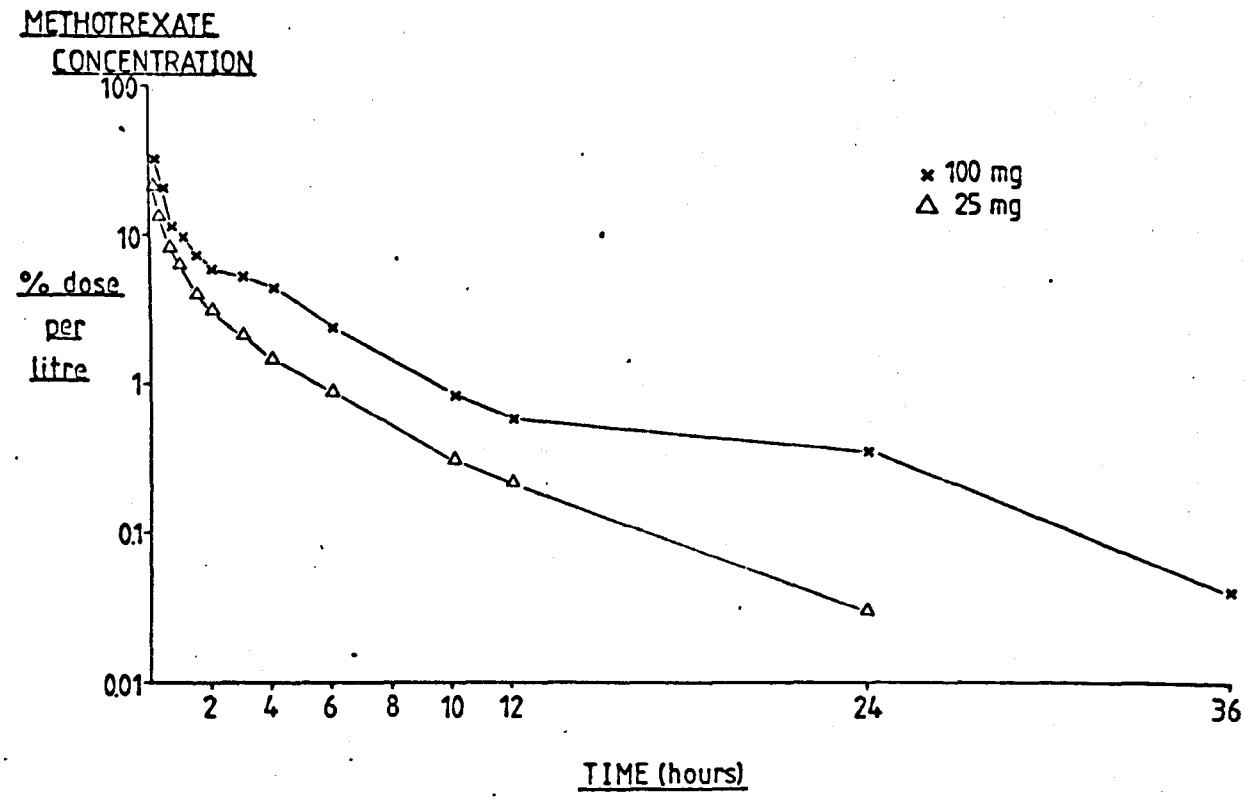


Figure 31 Mean methotrexate serum concentration/time curves after 100mg. and 25mg. i.v. bolus injection.

Drug levels are expressed in terms of % dose per litre, a normalisation which should result in super-imposable curves if the drug's pharmacokinetics are linear (Wagner, 1975). Drug levels so expressed are consistently higher following the 100 mg. dose from 1.5 h. after dosing (Table 26).

As a result of this divergence in serum concentration-time curves $AUC_{0-\infty}$ after 100 mg. methotrexate bolus is disproportionately greater ($p = 0.05$) than $AUC_{0-\infty}$ after 25 mg. (Table 27). Similarly total MTX clearance is consistently lower at the higher dose ($p = 0.05$) as is renal clearance of the drug. Recovery of MTX in urine up to 48 h. was 85 $\pm$ 11% after the 25 mg. dose but only 57 $\pm$ 17% following 100 mg.

While the total and renal clearance values quoted are not directly comparable due to the difficulties outlined above, inferred extra-renal clearance of the drug is small and does not change consistently with increased dose.

Patient	Dose	AUC $0-\infty$ (ng h ml^{-1})	Clearance (ml min^{-1}) Total $0-\infty$	Clearance (ml min^{-1}) Renal 0-48 h.	Inferred extra-renal
A. McG	25 mg.	7,380	56	55	1
	100 mg.	67,375	25	11	14
W.T.	25 mg.	7,465	56	39	17
	100 mg.	67,030	25	9	6
M.T.	25 mg.	9,677	43	42	1
	100 mg.	60,871	27	22	5
M. McI	25 mg.	8,655	48	39	9
	100 mg.	41,723	40	24	16
V.L.	25 mg.	6,044	69	54	15
	100 mg.	54,975	30	23	7
J.G.	25 mg.	4,293	97	89	8
	100 mg.	44,327	38	19	19
Mean ±S.D.	25 mg.	7,252 ± 1,904	62 ± 19	53 ± 19	9 ± 7
	100 mg.	56,050 ± 11,090	31 ± 6	18 ± 6	11 ± 6

$$\frac{\text{Ratio of } AUC_{0-\infty} \text{ after 100 mg.}}{AUC_{0-\infty} \text{ after 25 mg.}} = 7.7$$

Table 27 The AUC and clearance values of the individual patients after 25mg. and 100mg. i.v. bolus injection.

DISCUSSION

Goldie, et al., (1972) reported observations consistent with saturable methotrexate elimination when they suggested that a dis-proportionate increase in plasma methotrexate concentration followed infusion of a 20 g. dose. That others workers using comparable methotrexate doses (Stoller, et al., 1975) did not observe this phenomenon probably simply reflects inter-individual variation in methotrexate clearance. Within patient comparisons led Leme, et al., (1975) to incorporate a saturable renal elimination process in the model they proposed to describe the handling of methotrexate in doses above 80 mg/m^2.
A modification of this model was later used by Reich, et al., (1977) to describe the handling of doses of methotrexate between 50 and 200 mg/kg.

Renal excretion constitutes the main route whereby methotrexate is eliminated (Shen and Azarnoff, 1978) and as much as 50% of the infused dose has been recovered in urine within 12 h. after high dose methotrexate (Pratt, et al., 1974; Stoller, et al., 1975). While Liegler, et al., (1969) estimated the renal clearance of methotrexate at between 144 and 217 ml min^{-1} these authors were working at steady state plasma levels of 90-200 ng/ml. Huffman, et al., (1973) reported a renal clearance of 78 ml min^{-1} following a single dose of 30 mg m^{-2} (resulting in plasma levels up to 1,000 ng ml^{-1}). This estimate is more in keeping with the findings of the present study (where maximum methotrexate serum concentrations were up to 6,000 ng ml^{-1} after 25 mg. and up to 40,000 ng ml^{-1} after 100 mg.). The preliminary studies cited by Shen and Azarnoff (1978) yielded net renal clearance estimates of 20-50 ml min^{-1} at plasma levels between 0.1 and 500 μg ml^{-1}. While, as these authors point out that the exact relationship between renal clearance and methotrexate plasma levels awaits clarification, the results presented here show that the effect of methotrexate's saturable renal elimination can become apparent after modest doses administered by bolus I.V. injection.

Methotrexate appears to utilise the general organic acid transport mechanism for its tubular secretion as demonstrated by reduced methotrexate clearance in the presence of salicylate and para-aminohippurate (Liegler, et al., 1969). Saturability of this renal tubular secretion of the drug is the likely mechanism for falling renal clearance of methotrexate with increased drug dose.

2.4.2. Biliary Excretion of Methotrexate

The biliary clearance of a number of drugs constitutes an important elimination process (Riggs et al., 1977; Spring, 1968; Caldwell et al., 1971). Varying amounts of drugs excreted in this way may undergo enterohepatic cycling and result in persistence of drug in the body. Such a cycling process may also be responsible for intestinal toxicity, such as that observed for indomethacin (Duggan, et al., 1975). Although little is known about the elimination of MTX by this route, particularly the quantity of drug excreted, radio-labelled tracer studies have indicated that 1-2% of the activity of an intravenously administered dose is excreted in faeces (Henderson et al., 1965; Huffman et al., 1973; Wan et al., 1974). This figure referred to the parent drug plus metabolites, and it was assumed that most of the drug was absorbed by the intestinal mucosa. Wan et al., (1974) have reported that only 4-6% of a total dose of 30 mg. m^{-2} is excreted in faeces after oral administration. Therefore, considerable enterohepatic cycling of MTX may occur after intravenous dosing since 1-2% may be recovered from the stool. In this study an estimate of biliary clearance was made by measuring MTX concentrations in bile obtained by T-tube drainage.

Patient History: A cholecystectomy had been performed in a patient for the relief of chronic cholecystitis and fibrosis of the gall bladder and a choledochal T-tube inserted. A breast lump was biopsied at that time which proved to be malignant (adenocarcinoma) and chemotherapy was initiated. Treatment consisted of 500 mg. 5-fluorouracil given intravenously followed 24 h. later by a 50 mg. bolus injection of MTX. The patient was also receiving Ampicillin (500 mg. q.i.d.) and flurazepam (10 mg.).

Sample collections: Bile was collected by T-tube drainage and the volume measured and the time interval noted.

A 24 h. pretreatment sample of urine and bile was collected, and a sample of serum obtained immediately before MTX was given. Samples were collected at appropriate times until 75 h. post drug administration. All samples were stored at -20°C. until they could be assayed. Recovery of bile by T-tube drainage is not complete, but was assumed to be 65% efficient as reported in the literature (Riggs et al., 1977). The values reported here have been corrected accordingly.

Methods: Drug levels in serum, urine and bile were measured by radioimmunoassay employing the ^{75}selenium label. Appropriate dilutions of the pretreatment bile and urine, depending on the dilutions of the samples, were added to the standard curve when the MTX in these fluids was being assayed. This procedure eliminated any non-specific cross-reactivity which might otherwise have influenced the measurement of drug levels in these body fluids.

Serum biologic half life ($t_{\frac{1}{2}}$) was measured from the slope of the log-linear regression line drawn through the terminal portion of the serum concentration time curve. Renal and biliary clearance from 0-75 h. was calculated from the relationship.

$$\text{clearance} = \frac{\text{Amount excreted in urine or bile}}{\text{AUC (i.v.)}}$$

The area under the serum concentration time curve (AUC) was computed by the Trapezoidal Rule.

Results: The disappearance of MTX from serum followed a triphasic pattern (Fig. 33). The serum biologic half life ($t_{\frac{1}{2}}$) of the final elimination phase was found to be 12.2 h. The area under the serum concentration time curve (AUC) from 0-75 h. was 7.32 $\mu g h\ ml^{-1}$.

Bile flow varied throughout the study, the mean value being 10.51 $\pm$ 6.46 (S.D.) ml. h^{-1} and the cumulative bile volume at 75 h. was 610 ml. (Fig. 32). The total amount of MTX excreted over 75 h. was 0.103 mg. or 0.205% of the MTX dose administered and the biliary clearance was 0.23 ml min^{-1}.

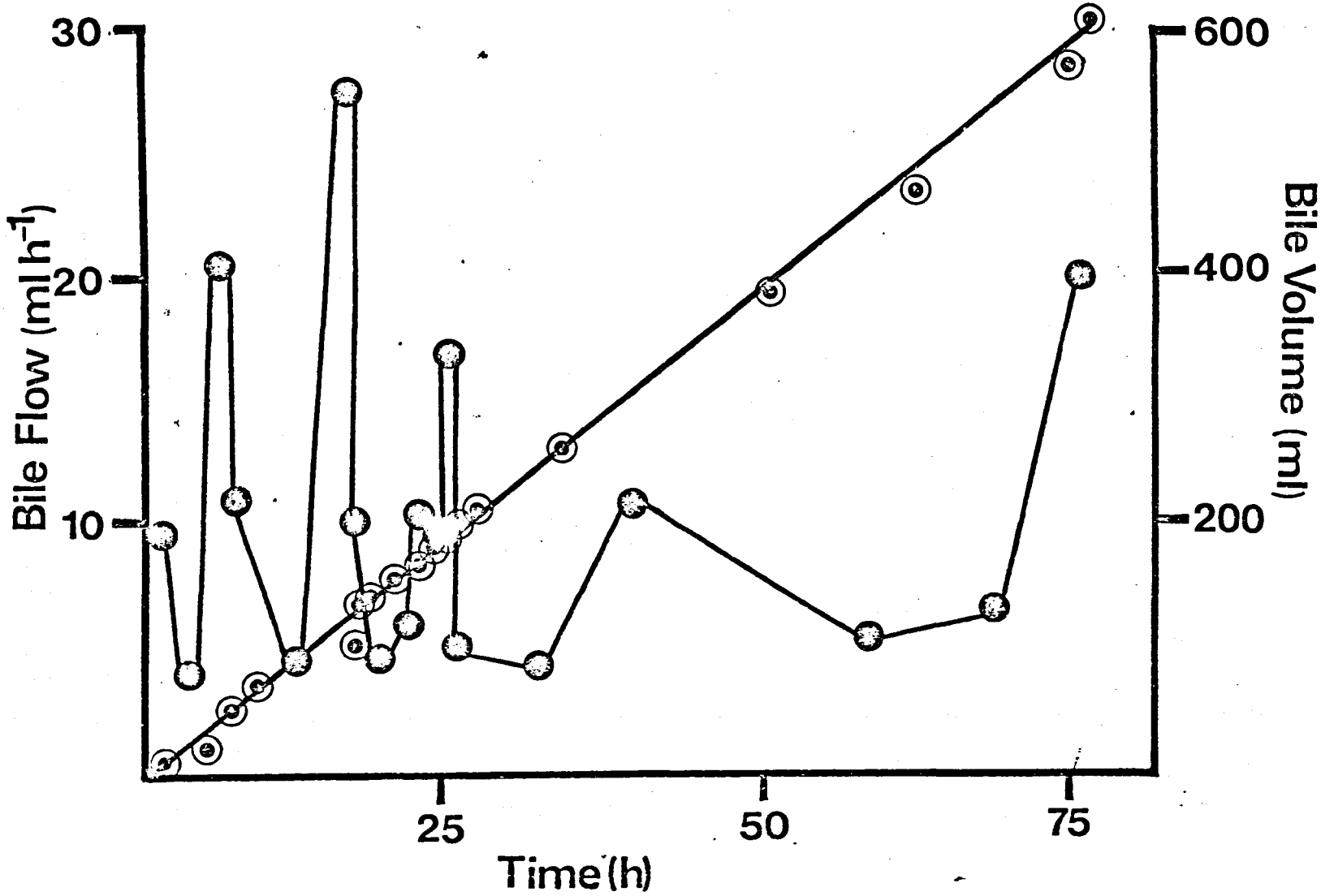


Figure 32 Bile flow(●) & cummulative bile volume(◉).

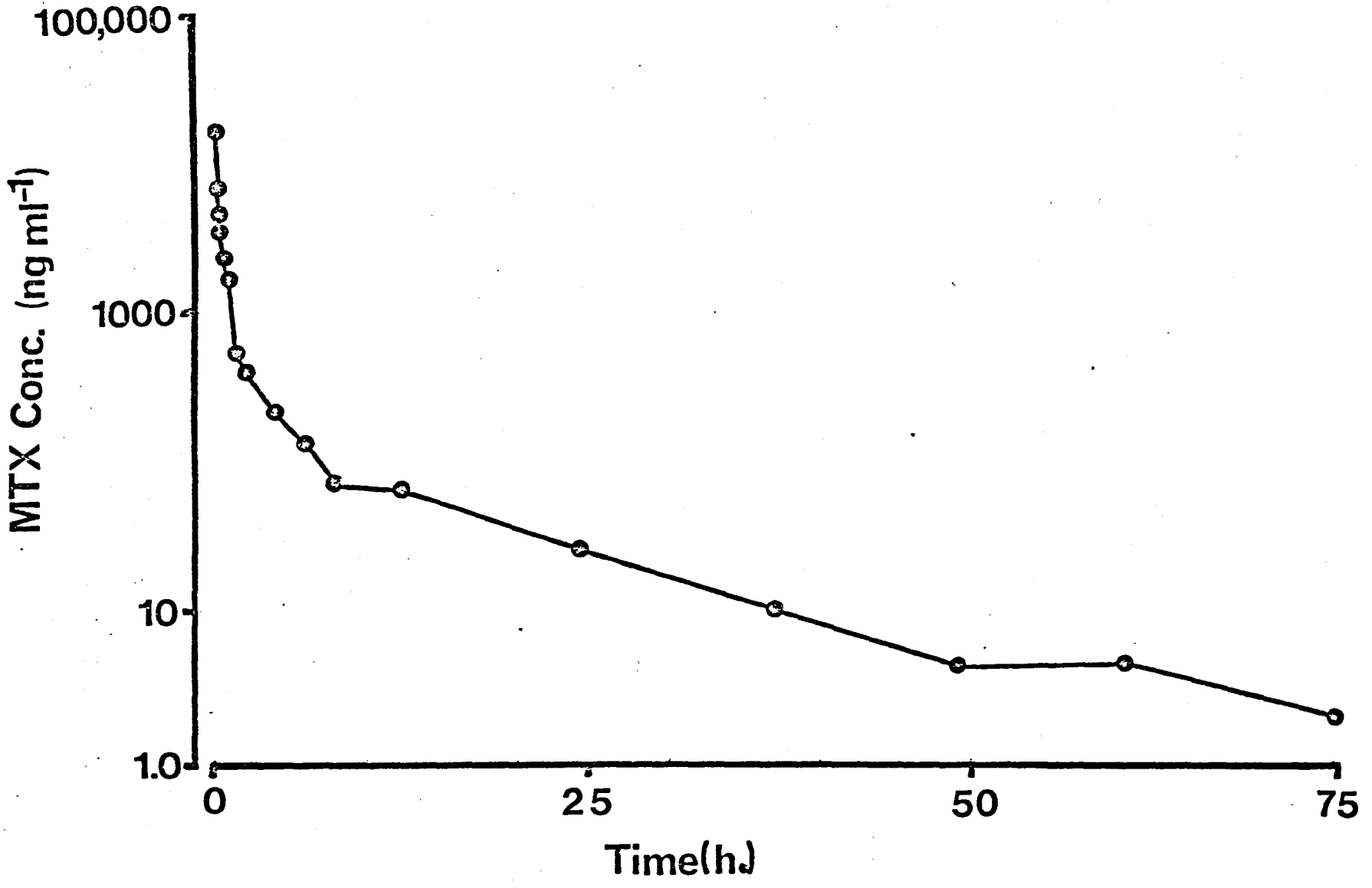


Figure 33 Concentration/time curve obtained after 50mg. methotrexate i.v. bolus.

The amount of MTX excreted in urine was 34.0 mg. or 68% of the MTX dose, and the renal clearance was 77.4 ml. min^{-1}. The biliary excretion : renal excretion ratio was 0.003 : 1 and remained constant over the time course of the experiment.

Discussion: This study indicated that biliary clearance is of minor importance compared to the renal clearance in the elimination of MTX. In view of the small amount of MTX in bile enterohepatic cycling would be negligible, certainly in terms of the effect of intestinal absorption of this quantity of drug on serum levels. However, the concentration in bile during the final elimination phase remains about six times greater than that observed in serum over this period of time. At 75 h. the concentration in bile is 2.2×10^{-8}M (serum concentration at this time being 0.44×10^{-8}M) which just exceeds the concentration threshold (2.0×10^{-8}M) necessary to produce toxicity in gastrointestinal epithelium reported by Young and Chabner (1973). This small but persistent quantity of MTX in bile may therefore contribute to the gastrointestinal toxicity which has been observed after intravenous administration.

The serum concentration decay profile and the biologic half life ($t_{\frac{1}{2}}$) is consistent with what is expected for MTX given in this dose by intravenous injection. There is, therefore, no obvious kinetic evidence to suggest that the biliary clearance figure found in this patient would be grossly different from that observed in any other subject. However, it is known that concomitant administration of other drugs can influence not only bile volume per se, but excretion of drugs in bile. Phenobarbital is perhaps that most striking example of this phenomenon (Bachur et al., 1974; Harris, 1976; Klassen, 1977), but this effect is produced predominantly by microsomal enzyme induction. Since MTX is not metabolised to any great extent when given intravenously (Anderson et al., 1970; Huffman et al., 1973; Wan et al., 1974), an effect produced by this mechanism is unlikely to be significant.

The radioimmunoassay used is extremely specific and does not cross-react with 7-hydroxymethotrexate (7OHMTX) but the assay has not been examined for cross-reactivity with polyglutamates which have been found in the liver (Jacobs et al., 1975; Rosenblatt et al., 1978).

and may be expected to be present in bile. Also since it was not possible at the time of this study to ascertain what quantity of polyglutamate metabolites were present, if any, it is assumed that the clearance value is that for unmetabolised MTX. The hepatic-biliary clearance is unlikely to be affected to any great extent by polyglutamates since they contribute only a small degree to the over-all metabolism of MTX (Jacobs et al., 1975). The cumulative bile volume (610 ml.) is in the range expected and would therefore suggest that no increase in bile flow had been promoted by the other drugs (5-fluorouracil, flurazepam, ampicillin).

CHAPTER 4

GENERAL DISSCUSION

Methotrexate has been used for the treatment of cancer for over thirty years but as yet there are certain aspects of its clinical pharmacology which remain unresolved. This present work describes studies which were undertaken to answer certain questions ingendered in response to clinical observations. It also sets out to clarify fundamental but ambiguous observations in the literature related to methotrexate bioavailability, protein binding and elimination. An attempt was made to relate the results of these studies to parameters of methotrexate therapy, such as efficacy, toxicity and resistance. The relevance to clinical practice was also discussed with some suggestions of how therapy may be modified to relieve patient stress.

A priori in any pharmacokinetic or pharmacological investigation is a specific and sensitive assay of high precision. The cross-reactivity of 7-OH MTX described in this thesis and the excellent work of Paxton et al., (1978) indicated a remarkable specific radio-immunoassay. Allied with the exceptionally low limit of detection and the high degree of precision, this assay becomes an excellent choice for measuring drug levels in body fluids. It ably facilitated the accurate measurement of pharmacokinetic parameters from drug levels in body fluids.

There was an extremely wide range in the values of kinetic parameters obtained in these studies. This was undoubtedly due in part to the nature of the disease in these patients. The values for the biological half-lives ($t_{\frac{1}{2}}$) recorded in the present studies were particularly widely scattered. It is critical that concentrations in plasma be measured for long enough to determine the true terminal half life. However, truncated data is sometimes unavoidable due to limitations imposed by the limit of detection of the assay. If the terminal biological half-lives between individuals were compared it is unlikely that such a wide scatter of values would be observed. For the purpose of the present studies individuals were compared according to the drug regimen and the ($t_{\frac{1}{2}}$) values compared from analysis of data collected over the same time course.

The detection limit of the radio-immunoassay enabled the measurement of methotrexate in plasma over several days. A reasonable estimate of the half life in this body fluid could therefore be made, even so it remains likely, since methotrexate persists in enythrocytes and probably in other body compartments, that the true terminal biologic half life is very much longer.

It was concluded that continuous ultrafiltration is a credible method for the study of protein binding, not only of methotrexate but of a number of other drugs. A satisfactory membrane could be found which would allow efficient separation of bound and free drug. Methotrexate was found to be binding predominantly to serum albumin. This binding was linear over a wide range in drug concentration, but as the serum concentration increase above 50 μ Mol. l^{-1} the binding became overtly non-linear. Such concentrations are only observed after high intravenous doses. Although the percentage bound methotrexate in patients did not differ significantly from an age and sex matched group, at higher concentrations the binding did appear to depend on the concentration of serum albumin. This was to be expected from the non-linear nature of the albumin/methotrexate interaction at high drug concentrations. Midler et al., (1950) observed that plasma albumin concentration varies inversely with the stage of the malignant process, progressively decreasing as the disease becomes more disseminated. Stenfeld (1960) also observed following eighty three patients with various types of cancer over a period of one month to five years, that serum albumin concentrations fell on average 10 g. l^{-1}. It is therefore to be expected that the percent methotrexate bound to plasma albumin would decrease with progression of disease. No difference could be demonstrated between controls and patients when the binding parameters were compared. It is also possible that anomalies may occur in some patients particularly if their disease is unchecked and albumin synthesis is progressively compromised.

*

The following sentence should be amended to read: "There was a significant correlation between drug concentrations in plasma and parotid saliva although such partition appears to be impeded as predicted from the physico-chemical properties of methotrexate".

It has been observed that some patients on methotrexate therapy show signs and symptoms of conjunctivitis and oral toxicity. Although the physico-chemical processes which determine the partition between plasma and saliva would tend to seclude methotrexate from secretions of the salivary glands very little information is available concerning these factors in tears. This is perhaps due to the fact that very few assay systems are sensitive enough to measure drug levels in tears, particularly as only small volumes can be readily collected. In any case there are few indications to measure drug levels in tears. It has been possible to demonstrate that methotrexate can distribute into tears and saliva. Distribution into tears appears to be dependent on the unbound levels in plasma, but no clear relationship between tear concentrations of drug and conjunctivitus could be derived. Folinic acid administration does appear to be effective in preventing the manifestations of this reaction suggesting that it is an effect dependent on methotrexate cytotoxicity rather than a purely chemical conjunctivitis. *Although there was a significant correlation between drug concentrations in plasma and parotid saliva it is clear, as expected, that methotrexate is secluded from secrection into saliva. The predictive value of determining plasma levels of methotrexate from that in parotid saliva is unfortunately poor. The collection of samples is time consuming an impractical particularly for the collection of samples from children where a painless and non-invasive method of monitoring plasma drug levels would be most acutely felt. Although no incident of oral toxicity was observed in these studies alterations in plasma protein binding of methotrexate resulting in increased levels of drug may precipitate this toxic reaction if the disease is not controled. It would be expected that the incidence of conjunctivitis may also increase for the same reasons.

The concentrations of methotrexate in C.S.F. or mixed saliva did not bear any clear relationship to the levels of drug in plasma.

There was a close corielation between drug levels in C.S.F. and the levels in mixed saliva which could be of great clinical potential. It may provide a rapid, simple,painless and non-invasive method of obtaining an estimate of C.S.F. concentrations of methotrexate. These results clearly indicate the need for further work to determine confidence limits. They also have wider implications however tenuous the proposition may be on this evidence, since there is the possibility that the transport mechanisms between plasma and C.S.F. and plasma and saliva are similar. In addition saliva levels may provide a simple way of monitoring C.S.F. levels of centrally acting drugs if the same relationship as that between the concentrations of methotrexate in saliva and C.S.F. can be confirmed.

Methotrexate enters and persists for long perioos, perhaps for several weeks within enythrocytes. The possibility exists that in so doing it may influence the expression of toxicity and resistance. The concentration time profile is consistent with a slowly exchanging kinetic compartment which does not appear to be influenced by folinic acid administration, at least not in an obvious manner.

It was demonstrated that the bioavailability of orally administered methotrexate as a syrup was dose dependent. This result was consistant with publish results by other workers. This problem was conviently overcome at least partly, by dividing high oral doses into units of 25 mg. given at two hourly intervals. Methotrexate formulated as a palateable syrup therefore permitted oral therapy to be considered as a possible alternative to the high dose intravenously mode. This has great advantages for the patient since treatment would be less traumatic and they could in some cases undergo treatment as an outpatient. It woud also help to relieve the strain on available facilities which often exists in busy oncology units.

Methotrexate elimination is predominantly by renal excretion although small quantities are excreted in bile. These small quantities encountered in bile suggest that enterohepatic cycling if it occurs would be negligible.

This small, but persistent, quantity of methotrexate may nevertheless contribute to intestinal toxicity particularly after high doses are administered. The results presented here also show that renal elimination of methotrexate is saturable, and that this effect can be apparent after modest doses administered by intravenous bolus. After high intravenous doses methotrexate may therefore persist in plasma for longer than expected and may increase the incidence of toxicity. Monitoring of plasma drug concentration is therefore advocated since increased doses of folinic acid or more prolonged rescue may be necessary.
A reduction in plasma albumin resulting in increased free levels of methotrexate may further complicate high dose therapy. Drugs such as analgesics or other cytotoxic agents which are likely to be given concomitantly could also displace methotrexate from binding sites and further increase the threat of severe toxicity.

APPENDICES

APPENDIX I

An example work sheet is illustrated for methotrexate absorption when given as 25 mg. x 4 (see page 100 for a description of the study and results). The work sheet show the features of all the studies reported in this thesis. Samples were collected for long periods and meticulous attention was paid to recording the exact time of sample collection. Due attention was also given to manifestations of toxicity and administration of other drugs.

Department of Materia Medica

University of Glasgow

Methotrexate : 25 mg. x 4

Name .....

Age ..... Sex ..... Wt (Kg) ..... Height (cm.) .....

Preparation ..... Route of administration .....

Date of Study .....

Diagnosis Histology:

Primary site:

Metastases: 1

2

3

History (circle) - Liver disease Renal Disease Smokes 0 + ++

Blood disease Other: Alcohol 0 + ++

Blood : Laboratory Report:

Hb	Urea	Creatinine or Creat.clearance
Bilirubin	Alk.Phosphatase	
Albumin	GT	ALT
		AST

	Drug	Dose	Route
Other Drugs (in week preceding study)			
Concurrent Drugs			

Comments: e.g. Nausea, Vomiting, Allergy.

Elapsed time	Ideal time	Event	Time done
		Light breakfast	
		Insert i.v. cannula ("Venflon")	
		Withdraw blood for control, Biochemical calues (20 ml. clotted)	
0 (START)		Administer first dose of drug orally (= time zero) (25 mg. Methotrexate)	
+ 10 min.		5 ml. blood (plain tube)	
+ 20 min.		5 ml. blood	
+ 30 min.		5 ml. blood	
+ 45 min.		5 ml. blood	
+ 60 min.		5 ml. blood	
+ 90 min.		5 ml. blood	
+ 120 min.		5 ml. blood	
		N.B. immediately before the second dose	

Elapsed time	Ideal time	Event	Time done
0 min		Administer 2nd dose of drug orally (= time zero) (25 mg. Methotrexate)	
+ 10 min.		5 ml. blood	
+ 20 min.		5 ml. blood	
+ 30 min.		5 ml. blood	
+ 45 min.		5 ml. blood	
+ 60 min.		5 ml. blood	
+ 90 min.		5 ml. blood	
+ 120 min.		5 ml. blood N.B. immediately before the third dose	
0 min.		Administer 3rd dose of drug orally (= time zero) (25 mg. Methotrexate)	
+ 10 min.		5 ml. blood	
+ 20 min.		5 ml. blood	
+ 30 min.		5 ml. blood	
+ 45 min.		5 ml. blood	
+ 60 min.		5 ml. blood	
+ 90 min.		5 ml. blood	
+ 120 min.		5 ml. blood N.B. immediately before the fourth dose	

Elapsed time	Ideal time	Event	Time done
0 min.		Administer 4th dose of drug orally (= time zero) (25 mg. Methotrexate)	
+ 10 min.		5 ml. blood	
+ 20 min.		5 ml. blood	
+ 30 min.		5 ml. blood	
+ 45 min.		5 ml. blood	
+ 60 min.		5 ml. blood	
+ 90 min.		5 ml. blood	
+ 120 min.		5 ml. blood and remove cannula	
12 h. FROM START		5 ml. blood	
24 h.		5 ml. blood	
36 h.		5 ml. blood	
50 h.		5 ml. blood	

ALL BLOOD SAMPLES SHOULD BE SEPARATED AS SOON AS POSSIBLE AFTER WITHDRAWAL AND THE SERUM STORED IN THE DEEP FREEZE.

APPENDIX II

NORMAL BIOCHEMICAL VALUES

The Normal Range of Some Relevant Serum Biochemical Values

Creatinine	60 - 120 μMol. l^{-1}
Creatinine clearance	90 - 120 ml. min^{-1}
Total Protein	60 - 75 g l^{-1}
Albumin	36 - 52 g l^{-1}
A.S.T. (aspartate transaminase)	13 - 42 iu l^{-1}
A.L.T. (alanine transaminase) .	11 - 55 iu l^{-1}
Bilirubin	5 - 20μMol. l^{-1}

APPENDIX III

LIST OF PUBLICATIONS

AND

COMMUNICATIONS TO SOCIETIES

PUBLICATIONS

STEELE W.H., BOOBIS S.W., MOORE M.R., GOLDBERG A., BRODIE M.J., SUMNER D.J. (1978). Protein binding of salicylate in cutaneaus hepatic porphyria. European Journal of Clinical Pharmacology. 13:309 - 313.

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BAIRD G.M., LAWRENCE J.R., STEELE W.H., WILLOUGHBY M.L.N., Non-invasive monitoring of CSF methotrexate (MTX) levels in children: A pilot study. B.P.S. Aberdeen 10 - 12 Sept. (1980).

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BAIRD G.M., CALMAN K.C., M^{c}VIE J.G., STEELE W.H., STUART J.F.B., WHITING B., Methotrexate radioimmunoassay studies in plasma, saliva and tears. B.P.S. London 4th - 6th January (1978).

CALMAN K.C., LAWRENCE J.R., M^{c}VIE J.G., SNEADER W.E., STEELE W.H., STUART J.F.B., Methotrexate kinetics: effect of subdividing an oral dose. B.P.S. London 3rd - 5th January (1979).

E.O.R.T.C. Pharmacokinetics and Metabolism Group. Brussels January (1978).

1. Pharmacokinetics of oral methotrexate syrup. M^{c}VILE J.G., SNEADER W., STEELE W.H.
2. Oral toxicity and conjunctivitis induced by methotrexate. STUART J.F.B., STEELE W.H., LAWRENCE J.R., M^{c}VILE J.G.
3. Evolution of safe administration protocol for high dose methotrexate. BAIRD G.M., WILLOUGHBY M.L.N., STEELE W.H., WHITING B.

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