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THE EFFECTS OF VARIABLE DOSE METHOTREXATE INFUSION IN THE LABORATORY RAT

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CONTENTS

PRECIS

THE EFFECTS OF VARIABLE DOSE METHOTREXATE INFUSION IN THE LABORATORY RAT

Methotrexate has been used clinically in the treatment of human malignant neoplasms for nearly forty years. In recent times patients have received this drug in an attempt to improve the cure rate of squamous cell carcinoma of the head and neck. In this context methotrexate has been administered by infusion followed by the administration of an antidote, leucovorin, prior to surgery.

A number of clinical trials have been undertaken worldwide, to assess the effectiveness of induction chemotherapy regimes including methotrexate, in the treatment of head and neck squamous cell carcinoma. However, there is a paucity of experimental data which details the histological effects of methotrexate-leucovorin regimes, in relation to their effects on oral carcinoma and normal tissues, such as gastrointestinal tract. Since such data cannot easily be obtained in human studies, it was decided to explore aspects of these questions in an animal model.

An animal model (rat) was available in the Department of Dentistry which would allow relatively simple investigation of the effects of methotrexate and methotrexate-leucovorin infusions on both oral neoplasms and normal tissues at the same time. However, because of a lack of pre-existing toxicity data concerning the infusion model developed for this study, it was felt that the study of methotrexate on induced rat oral neoplasms should be preceeded by the establishment of appropriate parameters in normal animals. Accordingly, this report

describes the establishment of an intraperitoneal infusion technique for methotrexate and methotrexate-leucovorin in normal rats and records the clinical and histological findings in these animals following the infusion of various doses of the chemotherapeutic agent.

Rats were subjected to a 24 hour intraperitoneal infusion of methotrexate while confined in a restraining device. A series of toxicity trials were carried out in order to determine approximate doses for the LD 10 and LD 90 of methotrexate, when delivered to these animals by this method. Leucovorin was given to one group of rats following the LD 90 dose in order to rescue them from the toxic effects of the methotrexate.

Rats suffered various signs and degrees of toxicity including weight loss, anorexia and diarrhoea under the effects of the methotrexate. Jejunal tissue was taken from rats having received the LD 10 dose of methotrexate (Group 1), the LD 90 dose (Group 2A) and the LD 90 dose followed by leucovorin (Group 2B).

Microscopic examination of Group 1 tissue revealed degenerative changes in the mucosal tissues which were most severe 48 hours after the end of the infusion period. By 96 hours a considerable recovery in small intestinal mucosal structure had occurred. Group 2A tissues demonstrated a progressive degeneration of the mucosa, culminating in ulceration and irreversible damage to the mucosa. Examination of Group 2B specimens again revealed a degeneration of the mucosa which was most severe 24 hours after the end of the infusion period. After 6 further days, tissues were again of normal appearance. Mitoses were observed throughout the sections in this group. This was interpreted as evidence

that leucovorin had rescued cells in the crypts of Lieberkuhn from methotrexate induced blockage of DNA synthesis.

It was concluded that the model developed would be appropriate for the study of the effects of methotrexate on induced oral tumours.

DECLARATION

This thesis is submitted in part fulfilment of the requirements for the Degree of Master of Dental Surgery in the University of Adelaide.

This thesis contains no material which, except where due mention is made, has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

M. Doddridge

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CHAPTER I

INTRODUCTION

History

Folic acid antagonists received their first clinical trials in 1948 when Farber confirmed the effectiveness of aminopterin for the destruction of leukaemic cells in children (Farber et al 1948). This major advance in cytotoxic therapy was stimulated by studies of the vitamin folic acid, which had been isolated during investigation of the disease megaloblastic anaemia. It was determined that yeast autolysate and crude liver extract would prevent this disease and that the active ingredient in these solutions would also prevent a similar disease in monkeys, characterised by mouth ulcers and leukopenia (Rall 1963). In 1940 a substance was isolated from Lactobacillus casei which, although chemically related to liver extract, was capable of causing bone marrow toxicity. When neoplasms in experimental animals were challenged with this extract, it was found that S180 tumour and mouse breast cancers were inhibited (Leuchtenberger et al 1944, Leuchtenberger et al 1945). This active substance was later identified as an antimetabolite of folic acid namely, pteroyl-triglutamic acid (Farber 1947). Farber et al, (1949) later showed that folic acid analogues had the effect of accelerating the course of acute leukaemia.

Folic acid was identified and first synthesised at Lederle laboratories in 1945 and shortly after, the antagonists aminopterin and amethopterin (methotrexate) were also synthesized (Seeger, Smith and Hultquist 1947, Seeger et al 1947). Following initial trials with teropterin, diopterin and 7-methylpteroic acid against solid malignant neoplasms and acute leukaemia, aminopterin was confirmed to be the most

active inhibitor. The first child, suffering from leukaemia, was treated with aminopterin in December 1947. Farber was able to report on profound though temporary remissions in his patients (Farber et al 1948). Methotrexate became available in 1949 (Farber 1949) and rapidly replaced aminopterin owing to its superior therapeutic index.

In the years following these pioneering studies, methotrexate has gained an established role in the treatment of acute lymphocytic leukaemia, non-Hodgkin's lymphoma, osteosarcoma, choriocarcinoma, breast cancer, small cell carcinoma of the lung and head and neck carcinoma. It has been administered to control severe psoriasis and in preparation for allogenic bone marrow transplantation.

Progress towards the use of methotrexate in the treatment of these diseases was slow. Difficulties were encountered with the purification of the drug and in monitoring of drug levels in patients. Nevertheless, methotrexate was responsible for the first reported cures of malignancies achieved entirely by the use of drugs when cases of choriocarcinoma and Burkitt's lymphoma were treated (Li, Hertz and Spencer 1956, Burkitt 1967). It was only in the late 1960's and early 1970's that activity was confirmed against lymphosarcomas and carcinomas after Djerassi introduced the practice of leucovorin rescue (Djerassi et al 1968, Djerassi et al 1972).

STRUCTURE AND PROPERTIES OF METHOTREXATE

Methotrexate is 4-amino-4-deoxy-N10-methylpteroyl-L-glutamic acid. Its structure is analogous to that of folic acid (Fig. 1.1), but an

amino group replaces the hydroxyl group in the pteridine ring and the N10 position is methylated.

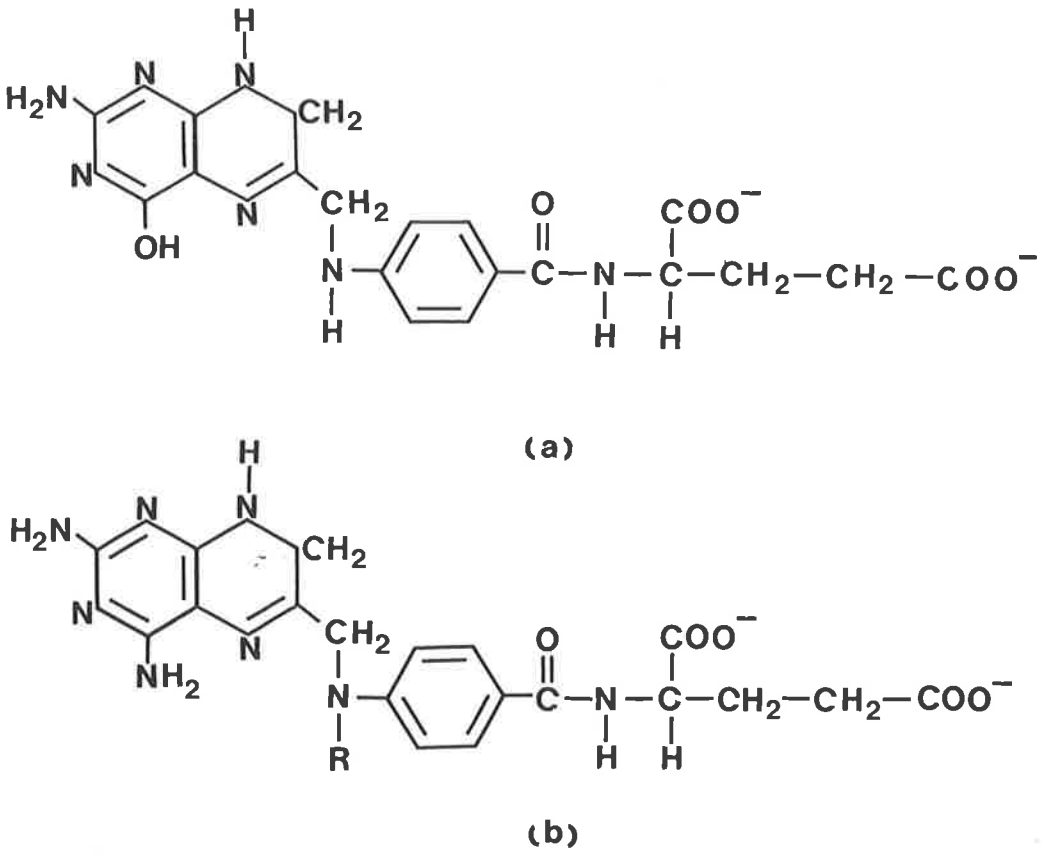


Fig 1.1 (a) Folic Acid
(b) Aminopterin (R=H) and methotrexate ($R=CH_3$)

A number of similar chemicals bearing 2,4, diamino groups also possess antifolate properties akin to those of methotrexate. There is no evidence that any of these have therapeutic advantages over methotrexate and familiarity with the older drug currently militates against its replacement.

Commercially available methotrexate contains a number of impurities which constitute up to 15% of the drug volume (Highnite, Shen and Azarnoff 1978, Chatterji, Frazier and Galetti 1978). The possible role of these impurities in causing drug toxicity is unknown (Chabner 1982).

Chemistry

The molecular weight of methotrexate is 454. It is a weak carboxylic acid which is ionised and lipid insoluble at physiologic ph. The drug will come out of solution and precipitate at high concentrations in acid solutions, a phenomenon which is sometimes encountered clinically in the kidneys.

Absorbtion and Distribution

Oral absorbtion is complete and predictable in low doses (Appendix I) (Henderson, Adamson and Oliverio 1965), but, higher doses are incompletely absorbed. This is believed to be caused by the saturation of an active transport mechanism. In the rat jejunum absorption is completely inhibited by folate or tetrafolate cofactors (Strum 1977). The drug is usually administered by other routes in both man and animals where the intravenous, intra-arterial, intrathecal, intraperitoneal, intramuscular and subcutaneous routes have all been the subject of investigation.

Zaharko et al (1971) developed a model which predicted the fate of methotrexate for 4 hours after administration in mice. They found that the drug was primarily eliminated by renal excretion and that there is an active enterohepatic circulation. A portion of the drug dose is metabolised by the intestinal flora. There are multiple drug half lives, the longest being of 3 hours duration. Each of these features has been observed in man. After intravenous injection methotrexate redistributes rapidly into extracellular fluids. Its disappearance from plasma can be described as triphasic (Stoller et al 1975). The initial half-life is

attributable to excretion and the drug entering the tissues. A second half-life is a consequence of excretion and metabolism of the drug. The third phase results from the enterohepatic recirculation of the drug and its release from cells (Huffman et al 1973). This terminal period can contribute considerably to both the bone marrow and gastrointestinal toxicity of methotrexate (Chabner and Young 1973). During this period plasma levels may still be high enough to cause gastrointestinal damage, but, succeptible cells can be rescued if an antidote to methotrexate is given. Methotrexate is concentrated in the liver and further concentrated in the bile. Much of the drug is then reabsorbed in the intestine.

In serum most methotrexate is bound to proteins, principally albumin. At high concentrations, this binding vehicle becomes saturated and free drug levels increase (Steele et al 1979). Distribution into interstitial fluid spaces, such as cerebrospinal fluid and interstitial spaces such as pleural and peritoneal cavities, occurs slowly, by passive transport (Dedrick et al 1975).

Organ distribution of methotrexate is directly correlated with the presence or absence of specific transport systems (Zaharko and Dedrick 1977). Methotrexate is rapidly transported into kidney, liver, skin and intestinal mucosa (Anderson et al 1970).

Mechanism of Action

The intracellular action of methotrexate is an example of competative inhibition. By tightly binding to the enzyme dihydrofolate reductase, it is able to prevent the conversion of folic acid to reduced

folate cofactors. Reduced folates are necessary for the metabolic transfer of one carbon unit in a variety of reactions contributing to both purine and pyrimidine synthesis.

N10 formyl-tetrahydrofolate and N5-10 methylene tetrahydrofolate contribute their labile one carbon groups in the synthesis of inosinic acid, the precursor of adenosine monophosphate (AMP) and guanosine monophosphate (GMP). N5-10 methylene tetrahydrofolate provides the methyl group and two hydrogen atoms for the synthesis of deoxythymidylate (dTMP) from deoxyuridylate (dUMP). Dihydrofolate is the product. Tetrahydrofolate is regenerated by the reduction of dihydrofolate and it is this reaction which is catalysed by dihydrofolate reductase (Fig. 1.2).

By interfering with these reactions both DNA and RNA synthesis are impaired. In doses of methotrexate not requiring rescue, the toxic effects of the drug appear to be primarily due to the interference in DNA synthesis (Hoffbrand and Tripp 1972). Because of this, the drug is cell cycle dependent, acting primarily in S-phase. Methotrexate can also disrupt protein synthesis. Reduced cofactors are needed for the conversion of glycine to serine and of homocysteine to methionine. Of clinical importance is the fact that free (unbound) intracellular methotrexate must be present for DNA synthesis to be continuously inhibited (Goldman 1974). This is because dihydrofolate levels can rise greatly when methotrexate is blocking dihydrofolate reductase. At high levels the latter competes with methotrexate for its target enzyme. Mammalian cells appear to lack the capability to accumulate free methotrexate. In order to achieve a high intracellular level, a continuous extracellular pool of methotrexate must be available (Goldman 1977).

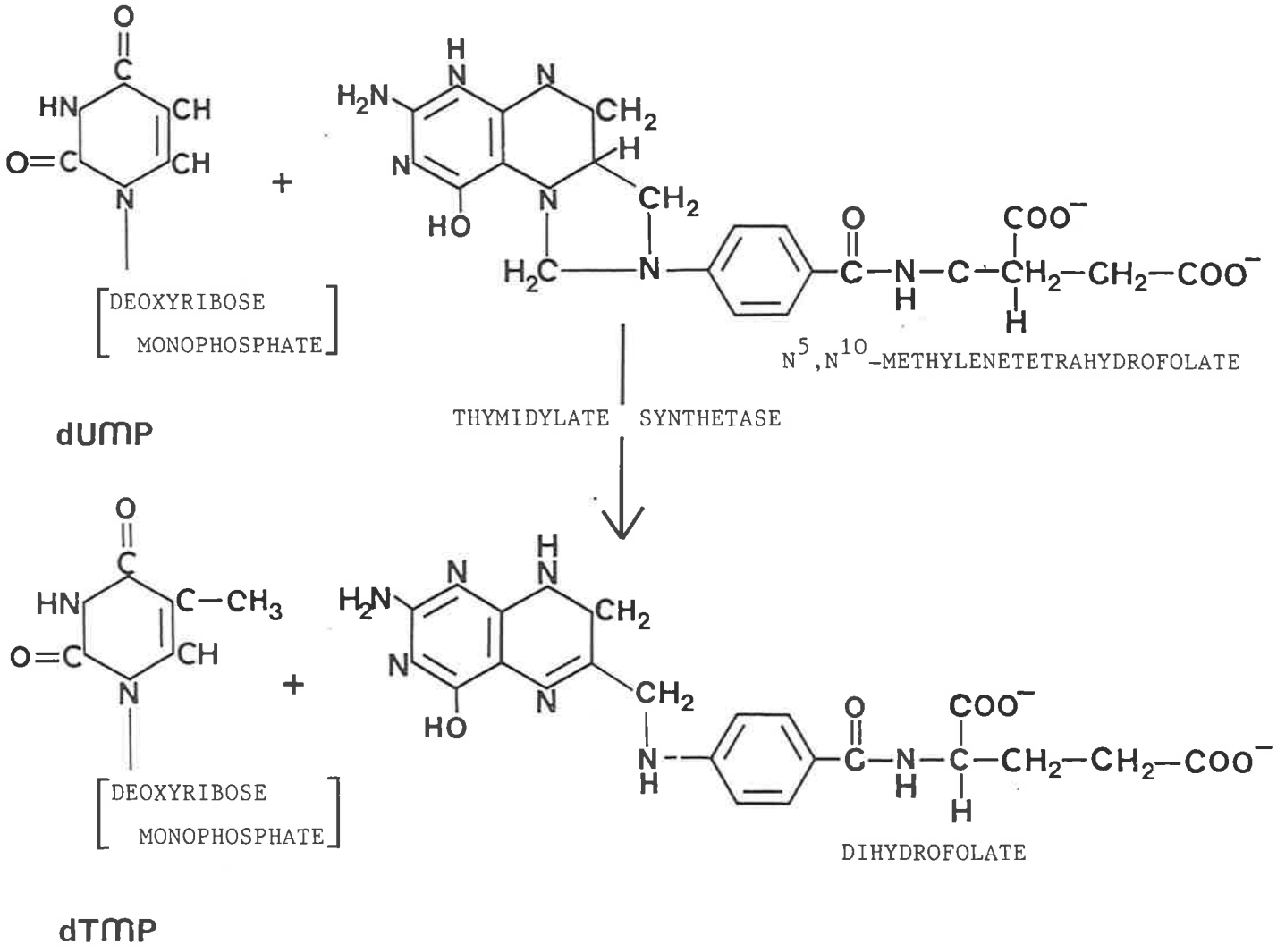


Fig. 1.2 The role of tetrahydrofolate in nucleic acid synthesis.

Methotrexate appears to enter both normal and neoplastic cells via a carrier mediated transport system (Goldman, Lichtenstein and Oliverio 1968, Goldman 1971a, White 1981). This energy dependent mechanism is shared by naturally occuring folates including 5-formyltetrahydrofolate (leucovorin). Therefore, methotrexate and reduced folates compete for entry into cells. Additionally, free intracellular methotrexate will efflux from cells if high concentrations of reduced folates enter a cell (Goldman 1971b). This has implications for the timing of rescue techniques during chemotherapy. It has been confirmed that methotrexate has greater affinity for the carrier in some malignant cells than in normal gastrointestinal mucosa (Sirotnak and Donsbach 1976). This property could contribute to a greater toxicity against malignant cells than normal cells, owing to a greater persistance of drug within the former.

At very high serum levels of methotrexate, a second cell entry mechansim operates. The details of this process are unknown, but this system is not competative with reduced folates (Warren, Nichols and Bender 1978). The eventual identification of this system may provide a rationale for the use of methotrexate in very high doses.

Transport of methotrexate is favoured when a cell population is rapidly proliferating (Chello, Sirotnak and Dorick 1980). This may contribute to the greater cytotoxicity seen when the drug is directed against rapidly dividing cells, but of course, these cells are already succeptible to methotrexate owing to a high rate of DNA synthesis.

Metabolism

It was originally thought that there was no significant metabolism of methotrexate. Recently, the use of high doses of methotrexate and the introduction of high pressure liquid chromatography has seen the detection of two notable metabolic products, 7-hydroxymethotrexate and 2-4-diamino-N10-methylpteroic acid (DAMPA). 7-hydroxymethotrexate is produced in the liver by the enzyme aldehyde oxidase. It is an effective inhibitor of dihydrofolate reductase but it is significantly less soluble than methotrexate. The poor solubility may contribute to renal toxicity (Jacobs et al 1976). DAMPA is a product of bacterial metabolism of methotrexate in the intestines by carboxypepsidase. DAMPA is a poor inhibitor of dihydrofolate reductase when compared with methotrexate. Another series of derivatives are the poly-γ-glutamates. These have been detected in human liver for months following the administration of methotrexate (Jacobs, Derr and Johns 1977) and have also been detected in rodent tissues (Whitehead 1977). The role of these metabolites (if any) in drug toxicity or activity is not yet understood.

Excretion

Methotrexate is rapidly excreted by the kidneys. In man 90% of an intravenous dose is excreted unchanged after 24 hours. At high plasma levels methotrexate is filtered and also actively excreted by the renal tubules. At low levels, evidence suggests that it is re-absorbed (Huffman et al 1973). Fecal excretion contributes in the order of 1-2% of an intravenous dose. Most of the drug excreted in the bile is re-absorbed in the intestinal mucosa (Bleyer 1978).

Methotrexate Toxicity

The critical sequel to the inhibition of dihydrofolate reductase by methotrexate is the depletion of reduced folate pools. The biosynthesis of both purines and thymidylate is consequently stopped and thus DNA and RNA synthesis is inhibited. In mouse bone marrow, a methotrexate concentration of 10^{-8}M is sufficient to block thymidylate synthesis (Pinedo et al 1977, Pinedo and Chabner 1977). Purine synthesis is blocked at a concentration of 10^{-7}M (Zaharko, Fung and Young 1977). Rodent gastrointestinal tract is even more succeptible to methotrexate toxicity. Chabner and Young (1973) found that intestinal crypt cells were not able to resume DNA synthesis until plasma levels of methotrexate had fallen to less than $5x10^{-9}$M.

The actual mechanism of cell death induced by methotrexate is not fully understood. Cessation of the production of DNA precursors inhibits DNA synthesis, but this may not directly kill the cells. DNA repair also is inhibited, but evidence suggests that this rarely results in cell death as conventional dose methotrexate has little effect on non-dividing cells (Chabner 1982).

Methotrexate is a phase dependant drug. It acts primarily in the S-phase of the cell cycle. This property would be expected to make the dose response curve terminate in a plateau, because only a portion of cells would be succeptible to the drug, despite an increased dose. However, the possibility that methotrexate may be toxic to cells out of S-phase is raised by the observation that high doses of methotrexate can inhibit slowly growing, solid tumours. In these circumstances the S-phase cells are obviously destroyed, but further cell kill may be caused

by the depletion of short lived proteins or the cessation of RNA repair capacity (Goldman 1977).

It would appear that tissue toxicity (and tumour cell kill) depends both on drug concentration and the duration of exposure to the drug. For each organ or tissue there is a minimum threshold concentration which must be exceeded before cells can be killed (Chabner and Young 1973). A minimum duration of exposure to levels above the threshold must also be attained.

The dose limiting toxicity factor for rodents is gastrointestinal damage. As previously mentioned, in rodent gastrointestinal tract toxicity to methotrexate is observed at lower drug concentrations than in bone marrow. The greater sensitivity of gastrointestinal epithelium is believed to be a consequence of the greater accumulation and persistance of the drug in this tissue (Chabner 1982). Rodent gastrointestinal epithelium can survive suprathreshold concentrations of methotrexate for a maximum period of approximately 24 hours. A lethal dose of methotrexate inhibits dihydrofolate reductase for more than 24 hours, after which cellular damage is such that renewal of epithelial cells ceases to occur (Sirotnak et al 1976, Sirotnak et al 1977, Zaharko and Dedrick 1973). If however, free methotrexate levels fall below the threshold and the time threshold has not been exceeded, mitotic activity in the surviving cells will recover.

The persistance of drug-enzyme complexes in the cell has no relationship to drug toxicity, because only a very low level of dihydrofolate reductase is necessary to maintain or restart DNA synthesis. Zaharko et al (1976) confirmed the concept of threshold drug

concentration by subjecting mice to low level methotrexate infusions for as long as two months. No signs of toxicity were manifest except minor facial hair loss.

In man, the time threshold appears to be about 42 hours (Levitt et al 1973, Young and Chabner 1973). This indicates that the tail or third half-life phase of methotrexate clearance is important in causing toxic effects. The severity of toxic changes is directly proportional to the duration of methotrexate exposure beyond the time threshold and relatively less dependent on the magnitude of elevation above the extracellular concentration threshold (Bleyer 1978).

In keeping with the expected effects on rapidly dividing cells, methotrexate toxicity in humans has its most dramatic effects on bone marrow cells and the epithelium of the gastrointestinal tract. Mucositis usually appears 3-7 days after drug administration and preceeds the fall in white blood cell numbers and platelet population by several days. Hepatitis and cirrhosis, intestitial pneumonia, alopecia and immunodepression are also seen (Thomas and Storb 1971, Nesbit et al 1976). At high doses, renal dysfunction, vomiting, acute desquamative dermatitis and β-lymphocyte dysfunction are among the problems encountered (Condit, Chanes and Joel 1969).

THE CONCEPT OF RESCUE

The toxic effects of methotrexate can be prevented by replenishing tetrahydrofolate pools with 5-formyltetrahydrofolate (leucovorin or citrovorum factor). This is an example of the concept of rescue, the utilization of a drug antidote to maintain the viability of normal

tissues selectively, following the administration of a cytotoxic agent. That this concept could be put into practice was established during the rodent experiments of Goldin and co-workers (Goldin et al 1953, 1954, 1955, 1966). The term was first used in the clinical context by Capizzi et al (1970) during studies of high dose methotrexate therapy for head and neck carcinoma.

Besides leucovorin, other agents are available which contribute to the rescue of cells from methotrexate toxicity. Carboxypepsidase G1 is an enzyme which is able to hydrolyze folate analogues, thus markedly reducing their ability to inhibit dihydrofolate reductase (McCullough, Chabner and Bertino 1971). Asparaginase is an enzyme which has potent antineoplastic properties against several experimental malignancies. As an inhibitor of protein synthesis, it is able to decrease the killing rate of agents such as methotrexate which cause thymineless death (Capizzi, Summers and Bertino 1971). Relief from methotrexate action can also occur by supplying the cell with products which are deficient because of the methotrexate blockage of dihydrofolate reductase. Thymidine can be used in this way (Tattersall, Brown and Frei 1975).

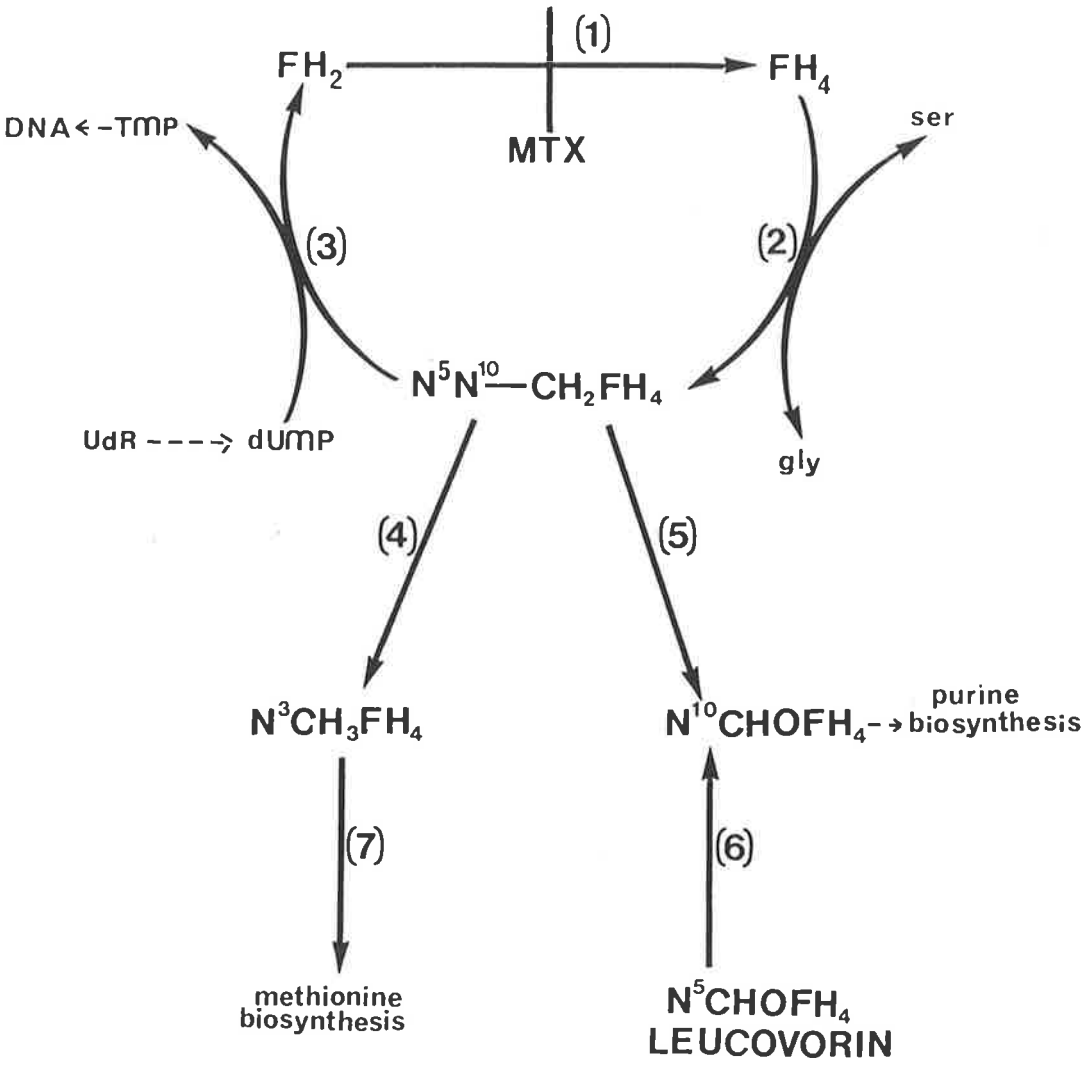
Pharmacology of Leucovorin

Leucovorin is available commercially as its calcium salt. There are two diasteromers of which only one is physiologically active (Nixon and Bertino 1971). Leucovorin is rapidly converted to 5-methyltetrahydrofolate and this compound is actually the rescue agent. Both unchanged leucovorin and 5-methyltetrahydrofolate are excreted in the urine. Oral absorbtion is rapid and reliable, but in the face of gastrointestinal toxicity, leucovorin should be administered parenterally. Leucovorin has

a half-life of 0.7 hours and 5-methyltetrahydrofolate has a half-life of 2.2 hours. Re-administration, or infusion of leucovorin, is usually necessary to encompass the period of potentially toxic serum methotrexate levels (Mehta et al 1978).

The biochemical basis for the rescue of cells by leucovorin from methotrexate induced DNA synthesis block, is the bypassing of the block by the supply of reduced folates into the cell, (Fig. 1.3). Additionally, delivery of methotrexate to the cell can be reduced by competition for the uptake of the drug. Rescue techniques can only increase the efficacy of a drug if they are able to rescue normal cells at the expense of the malignant cells. If malignant cells are rescued equally, the rescue agent will only act as an antidote and limit toxicity. The therapeutic effect will be lost and the therapeutic index will not be increased. Preferential rescue of normal cells can potentially occur if the mechanism of methotrexate induced cell death is different between the normal and malignant cell populations, or if the malignant cells are less able to allow the entry of the rescue agent at the expense of methotrexate, than are normal cells.

In mammalian cells, reduced folates can compete with methotrexate for transport into cells. If the ratio of extracellular leucovorin to methotrexate is high, influx of methotrexate into the cell is blocked. Efflux of methotrexate is coincidentally encouraged. The presence of reduced folates inside of the cell causes dissociation of the dihydrofolate reductase-methotrexate complexes. The free enzyme is then able to commence tetrafolate synthesis and finally DNA synthesis (Bertino 1977).



Abbreviations:

FH_2	dihydrofolate	FH_4	tetrahydrofolate
ser	serine	gly	glycine
TMP	thymidylate	UdR	deoxyuridine
dUMP	deoxyuridylate		

(1) Dihydrofolate reductase
(2) serine transhydroxymethylase
(3) thymidylate synthetase
(4) methylene tetrahydrofolate dehydrogenase
(5) N^5, N^{10} methylene tetrahydrofolate dehydrogenase
(6) N^5 formyl tetrahydrofolate isomerase
(7) methionine synthetase

Fig. 1.3 Leucovorin bypasses methotrexate induced tetrahydrofolate deficiency.

Two critical elements influence the outcome of rescue. Firstly, the dose of the rescue agent (as related to the dose of methotrexate), secondly, the timing of the rescue, in terms of the time of intervention and the duration of treatment with the rescue agent. Co-administration of a rescue agent with the drug is termed protection. This manoeuvre will reduce toxicity but decrease the efficacy of the drug.

Studies of the ability of leucovorin to rescue animals from the toxic effects of aminopterin were published as early as 1950 (Burchenal et al 1950, Schoenbach, Greenspan and Colsky 1950). Goldin and co-workers devised a series of experiments using mice innoculated with the transplantable leukaemia L1210 (Goldin et al 1953, 1954, 1955, 1966). Goldin reviewed this work in 1978 (Goldin 1978). He realized that the chance of curing an animal of a malignant disease is related to the number of neoplastic cells present. One cannot increase the dose of a drug such as methotrexate with impunity to counteract an increased neoplastic cell load, because toxicity becomes greater. Whilst leucovorin was expected on theoretical grounds to rescue cells from methotrexate toxicity, what needed to be established was the optimal timing of its use and whether it was possible to rescue normal cells at the expense of the malignant cells.

Goldin was able to show that the LD 50 of aminopterin was considerably higher when co-administered with folinic acid, but that the efficacy was reduced compared to that seen when aminopterin was used alone. When leucovorin was delivered 12-24 hours following aminopterin, the toxicity of the drug was reduced but the antileukaemic effectiveness was not. This regime was more effective in achieving tumour free survival in mice than methotrexate alone, or than methotrexate with

simultaneously administered leucovorin. When the use of leucovorin was delayed, survival was made possible despite a higher innoculum of leukaemic cells. Therefore, it was postulated that leucovorin has the potential to selectively rescue normal cells from the toxic effects of methotrexate. With concommitant administration of the two agents, protection was also afforded to the leukaemic cells.

Experimental evidence suggest that to achieve rescue, the dose of leucovorin needs to be sufficient to block further methotrexate entry into the normal cell and promote its efflux. If the level of methotrexate is very high, the dose of leucovorin may need to be one or two orders of magnitude greater to provide effective rescue (Esminger, Grindley and Hoglind 1979). Pinedo et al (1976) found that mouse bone marrow cells could only be rescued when serum methotrexate levels fall below 10^{-6}M. Administration of the rescue agent also needs to be maintained until the serum methotrexate level is less than 10^{-8}M, otherwise toxic effects occur during the slow final phase of drug clearance (Chabner 1982). This is because leucovorin has a short half-life compared to the terminal half-life of methotrexate (10.4 $\pm$1.8 hours in humans (Stoller et al 1975). The danger also exists of exceeding the optimal dose of leucovorin and subsequently rescuing the malignant cells as well as the normal cells. This phenomenon was reported by Esminger, Grindley and Hoglind (1979). In mice treated by 12 hour infusions of methotrexate followed by equimolar leucovorin, leukaemic cells were not killed. At 10% of the leucovorin dose, selective rescue occurred, but tumour cell kill continued.

Sirotnak et al (1977) found that DNA synthesis ceased in mouse small intestine one to two hours after injection of methotrexate.

Following an equimolar injection of leucovorin, a minor cycle of DNA synthesis was detected within one hour. At 4-6 hours the rate corresponded to 3-5% of the normal rate. After 19 hours a major cycle of synthesis was initiated in both leukaemic cells and small intestinal cells. Malignant cells were not killed. Giving leucovorin at a dose equal to 6% of an equimolar dose of methotrexate allowed DNA synthesis to recover to 25% of normal within 24 hours and completely by 40 hours. Under these conditions the leukaemic cells showed only very minor levels of DNA synthesis at 24 hours and cell kill continued. These experiments also showed that rescue by leucovorin can commence very rapidly following parenteral administration.

Whilst the optimal dosage and time schedules for the leucovorin methotrexate combination can be defined in small animals under specific experimental conditions, they have not been determined for humans. The value of the use of high-dose methotrexate with rescue has been debated. It appears to have been proven superior to conventional dose therapy against osteogenic sarcoma, non-Hodgkin's lymphoma and perhaps for head and neck carcinoma (Frei et al 1980). Several randomized studies of high-dose methotrexate with leucovorin rescue versus standard unrescued doses when used against head and neck carcinoma are available for scrutiny (Levitt et al 1973, Vogler et al 1979, Woods, Fox and Tattersall 1981, DeConti and Schoenfeld 1981) (Table 1.1).

These results have failed to confirm a clinical advantage in using high-dosage methotrexate. What is apparent from these studies is that although response rates were not significantly different, an increase in therapeutic index can be achieved if rescue is used following moderate

Investigator	Dose-Schedule		Evaluable Cases	Responses (CR + PR)	%	Drug related deaths
Levitt (1973)	MTX 80 mg/m^2 IV 30 hr infusion	2 weekly	16	7	44%	4
	MTX-LCV 240-360 mg/m^2 IV 36-42 hr -> 1080 mg/m^2		25	15	60%	2
Vogler (1970)	MTX-LCV 125 mg/m^2 po	6 hourly 4 times weekly	49	11	22%	1
	MTX 15 mg/m^2 po	6 hourly 4 times weekly	44	12	27%	1
	MTX 60 mg/m^2 IV	weekly	61	20	33%	1
DeConti (1981)	MTX- 40 mg/m^2 IV	weekly	77	21	27%	2
	MTX-LCV 240 mg/m^2 IV	weekly	74	19	25%	5
Woods (1981)	MTX-LCV 50 mg/m^2 IV	weekly	23	6	26%	1
	MTX-LCV 500 mg/m^2 IV	weekly	27	7	26%	0
	MTX-LCV 5000 mg/m^2 IV	weekly	22	10	45%	3

MTX - Methotrexate
LCV - Leucovorin

Table 1.1 Randomiized trials of methotrexate vs methotrexate-leucovorin rescue in squamous head and neck cancer modified from Pitman and Bertino (1984).

to low doses of methotrexate (Kirkwood et al 1981). The technique also remains of value in counteracting unexpected toxic effects of the drug and in making the course of chemotherapy more tolerable.

RAT INTESTINE: ANATOMY AND HISTOLOGY

According to Hebel and Stromberg (1976), the rat intestinal tract consists of the following structures:

	Length (mm)	Diameter (mm)
Duodenum	95-100	2.5-3
Jejunum	900-1350	4-5
Ileum	25-35	3-5
Caecum	50-70	10
Colon	90-110	10->3 (proximal -> distal)
Rectum	80	3-10

The duodenum arises from the distal end of the pylorus. The mucosa of the duodenum forms folds 1 mm high and up to 5 mm wide. Brunner's glands are found in the first few millimetres, in the submucosa. From the distal portion of the duodenum the intestinal submucosa is periodically dilated by Peyer's patches. These are masses of lymphoid tissue. Their diameter may reach 5 mm.

The small intestine is composed of four concentric structures. These are the mucosa, the submucosa, the muscularis and the serosa. The intestinal mucosa is specialized in order to perform the function of an absorbtive surface. It has multiple raised fingers called villi which extend the surface area of the intestine and multiple invaginations

called crypts of Lieberkuhn. The core of each villus is occupied by a specialized lamina propria.

The jejunum is the largest segment of the intestine. Its multiple villi are almost uniformly 0.6 mm long and are oriented at 90° to the long axis of the gut. The ileum contains villi arranged similarly to those of the jejunum. The villi of adjacent rows overlap each other. Villus size decreases gradually along the length of the small intestine. Villi can be as much as three times larger in the duodenum than in the terminal ileum (Altmann and Enesco 1967, Altmann and Leblond 1970).

Small Intestinal Epithelium

The villi are covered by a single layer of simple columnar epithelium which is constantly being renewed. The cells function as absorbtive cells and also secrete digestive enzymes. Three types of epithelial cells can be identified. These are the intestinal absorbtive cell, the goblet cell and the Argentaffin cell.

Intestinal absorbtive cells are of columnar shape with an ovoid nucleus at the basal end of the cell. The free surface has a prominant striated border which is composed of microvilli. The epithelium is covered by a protective layer of mucous lying on the microvilli. This is secreted by the goblet cells. These have a basophillic cytoplasm and basally located nuclei. Their goblet shape is due to distortion of the apex of the cell by secretory granules. Argentaffin cells can be found at the base of the epithelium between the columnar absorbtive cells. These are rounded or pyramidal cells with a granular cytoplasm. They are

believed to secrete seretonin which has a role in the process of peristalsis.

Crypts of Lieberkuhn

The base of the crypts of lieberkuhn are the site of intense mitotic activity. This area contains undifferentiated stem cells which differentiate into absorbtive cells and goblet cells as they ascend out of the crypt. The cells continue to divide at mid-crypt level but cease division as they move onto the villus (Neutra and Padykula 1983). After two days the new cells have been sloughed from the villus tip. According to the data of Leblond and Stevens (1948), the average time taken for a cell to migrate from the depths of the crypt to the tip of the villus is 32.4 hours. They also estimated that during the period of 37.7 hours 100% of the cells in the depths of the crypts of Lieberkuhn would undergo mitosis. This data was gained by studying adult albino rat ileum.

An additional cell type found in the crypts of Lieberkuhn is the paneth cell. Paneth cells are pyramidal in form with round or ovoid nuclei near the base and conspicuous secretory granules in the apical cytoplasm. These cells reside in the depths of the crypts but their exact function is not known. They are rarely seen in mitosis.

The crypts of Lieberkuhn are the sites of action of methotrexate in the gastrointestinal tract. The high mitotic rate in this area ensures a large target population of cells at all times for the drug.

Lamina Propria

The lamina propria forms the core of the villi and resembles reticular connective tissue. It contains a stroma of argyrophilic fibres with associated cells showing pale nuclei. Thin strands of smooth muscle extend into the core of the villi. They tend to be arranged around a central lymphatic vessel, the lacteal, which runs between the crypts of Lieberkuhn toward the submucosal lymphatic net. Each villus also contains an arteriole, which branches near the tip of the villus into a capillary network. One or two veins run into a venous plexus at the base of the villus. Lymphocytes, plasma cells, eosinophils and macrophages are all present. The most numerous of these tend to be the lymphocytes.

TOXICITY OF METHOTREXATE IN RATS

Cytotoxic drugs have traditionally been tested for toxicity and for efficacy in experimental animals. The rationale behind such studies is that drugs which are active in test animals will have a reasonable likelihood of being active against neoplasms in humans. For example, there can be a good correlation between effectiveness of methotrexate as an antineoplastic agent in animal models and human disease. The destruction of a population of leukaemic cells in mice was duplicated by using methotrexate to treat childhood leukaemia (Goldin et al 1959, Djerassi et al 1966). In other cases there has been a poor correlation between chemotherapeutic successes in the laboratory and in the clinical setting. One of the reasons for this is that common aetiology between animal models and human malignant neoplasms has not been demonstrated (Schabel 1975a).

In spite of this deficiency, animal studies have provided valuable data and demonstrated that there are pharmacological, biochemical and biological factors which influence the antitumour selectivity of a drug and its net therapeutic effect (Goldin and Schabel 1981). Variables such as drug dosage, schedule and route must be manipulated to achieve the maximum benefit from the drugs potential. The dose response curves of most cytotoxic agents are very steep, rising rapidly to a lethal toxicity. The survival time of an animal with a malignant neoplasm will increase with increasing dose until a lethal toxicity is exceeded. In evaluating the response of a tumour model to a cytotoxic drug, it is necessary to ensure that the tumour will only kill the animal after the toxic effects of the drug have subsided. There is then no controversy as to the mechanism of death. Early death is due to the drug, late death to the tumour.

The schedule of drug administration which is most effective may be closely correlated between the human and animal systems. It has been shown that rodent leukaemia responds better to intermittent high-dose treatment with methotrexate than to daily treatment. This principle has been successfully duplicated in the treatment of childhood leukaemia (Goldin et al 1956, Selaway et al 1965).

Data from animal experiments has further clinical relevance as there is a relationship between the toxic doses of cytotoxic agents in animals and in man. Freireich et al (1966) found a good correlation between the toxicity of 18 anticancer agents in man and in animals such as mice, rats hamsters, dogs and monkeys. The maximum tolerated dose per square metre of body surface area is very similar in man and each of the animal species.

The sites of toxicity tend to show a good correlation between laboratory species and man, as far as bone marrow, gastrointestinal tract, liver and kidney are concerned (Owens 1963).

The classic end point for assessing drug toxicity in small animals is death expressed as LD 10, LD 50, LD 90. LD 10 is not as reliable statistically as LD 50. However, LD 10 does correlate very well with the maximum tolerated dose in humans (Freireich et al 1966), so the toxic effects of a LD 10 dose of drug in an experimental tumour system is likely to best predict the effect of a drug trial against a comparable human tumour.

The toxic effects of methotrexate depend on the duration of DNA synthesis inhibition produced, rather than the total dose given. For this reason, daily small doses of the drug are much more toxic than are single administrations, which comprise the same total dosage.

The toxicity of aminopterin was studied in rodents soon after its discovery. Research extended to amethopterin on its introduction. The effects were naturally similar, but aminopterin was found to be more toxic at a given dose. Ferguson, Thiersch and Philips (1950) determined that the LD 50 of a single intraperitoneal dose of methotrexate varies between 6 and 25 mg/kg in rats. These animals were small (80-100 gm) and the purity of the drug at this time was poor. Dividing the dose into 5 equal daily fractions decreased the LD 50 to 5.6 $\pm$1.7 mg/kg (in total). Rall (1963) mentioned that the LD 50 for a single dose was 100 mg/kg but that if the drug was delivered daily for 15 consecutive days, the LD 50 was reduced to 0.25 mg/kg/day.

Sindram, Snow and VanPutten (1974) delivered methotrexate in a continuous intra-arterial infusion for 7 days. By this method the LD 50 was determined to be 2.1 mg/kg/day.

Taminiau, Gall and Hamilton (1980) injected a single dose of methotrexate of 30 mg/kg into rats stating that this dose approximates the LD 50. None of these animals was noted to have perished despite being observed for as long as 8 days.

Such variability in the toxicity data suggests that it would be unwise to extrapolate results from one experiment in designing another if the size or type of rat is changed, or if the method or schedule of drug administration is varied.

As indicated previously, the dose limiting body system in rodents appears to be the gastrointestinal tract (Chabner and Young 1973). Animals succumbing to acute toxic effects of methotrexate die following acute diarrhoea (Robinson, Antoniolo and Vanotti 1966), or secondary infection in the intestines. This later sequelae is mentioned by Philips and Thiersch (1949) who noted salmonella infection in 5% of their rats, which eventually led to the animals death.

Behavioural changes and symptoms are usually noted from 24 hours following administration of methotrexate by any route. In animals subjected to a lethal dose, severe disturbances are seen during the second day and continue until the death of the animal. Anorexia is a significant finding, observed within 24 hours of drug administration (Ferguson et al 1950, Millington et al 1962a, Loehry and Creamer 1969, Taminiau et al 1980). Terminally ill animals fail to regain their

appetites. Animals show malaise on the day following injection and lethargy and weakness supervene, the animal often assuming a hunched position (Woll and Olsen 1951). Alopecia does not appear to be a feature of methotrexate toxicity in rodents, although they have been noted to exhibit a ruffled fur. Pigmentation is seen about the eyelids, paws, tail and snout (Woll and Olsen 1951, Millington et al 1962a, Loerhy and Creamer 1969). Diarrhoea occurs during the second post injection day and its continuation for several days is indicative of severe or morbid toxicity (Ferguson et al 1950, Vitale et al 1954). Anorexia and diarrhoea leads to dehydration and progressive weight loss. Ferguson's (1950) terminally ill animals had lost 20% of their body weight and appeared dehydrated and dyspnoeic. Millington et al (1962a) found that experimental animals which were recovering from a dose of 5 mg/kg of aminopterin produced solid stools by the fifth day and this coincided with a return of their appetites and the beginings of a rise from the trough level of their body weights.

A sublethal dose of methotrexate can be expected therefore, to cause transient symptoms of toxicity and weight loss from which the animal will recover after several days. A lethal dose results in earlier, more severe and irreversible disturbances, characterized by anorexia, diarrhoea and weight loss, from which the animal fails to recover. In these circumstances, most deaths occur during the third or fourth post administration day.

On examination of the gastrointestinal tract some gross changes are identifiable. Robinson et al (1966) and Millington et al (1962a) reported that the small intestine became shorter in length and increased in diameter. The lumen of severely intoxicated rat contained a foul

smelling liquid and some haemorrhagic elements. Ferguson et al (1950) also noted enlargement of the liver and spleen and mesenteric lymphadenitis in their animals.

Histopathological Effects of Methotrexate on the Small Intestine of Rats

A number of investigators have studied the intestines of the rat following the administration of methotrexate or aminopterin. These studies have had a number of aims. The early works of Philips and Thiersch (1949) and Ferguson in collaboration with the same authors (1950), recorded acute toxic effects of the drug and regarded these as evidence that 4-amino folic acids are antagonists of folic acid. The action of methotrexate was investigated with regard to the pathogenesis of malabsorbtion syndromes such as sprue by Woll and Olsen (1959), Small et al (1959), WynnWilliams (1961) and Millington et al (1962b). Others have studied the histology and cellular dynamics following various doses of methotrexate (Millington et al 1962a, Robinson et al 1966, Loerhy and Creamer 1969, Taminiau et al 1980). The effect of methotrexate on the small intestine have also been compared with those of ionizing radiation, by Altmann (1974). He was able to report that the histological picture is remarkably similar following the two treatments.

The drug has been given by a variety of routes and in various regimes by these investigators. None of the studies used a continuous infusion as a means of drug delivery. The degree of tissue disturbance naturally reflects the doses used (Table 1.2).

Some investigators have subjected all of the gastrointestinal organs to scrutiny. In these studies, few or no microscopic changes were

Author	Drug	Dose	Route	Regime	Toxicity (and authority quoted)
Ferguson (1979)	MTX	50 mg/kg	IP	Single	Lethal
Philips (1950)	AMINOP	5-20 mg/kg 10-40 mg/kg	IP Oral	Single	Lethal
Woll (1951)	AMINOP	210 mg/kg 850 mg/kg	Oral Oral	Daily Single	Lethal Lethal
Vitale (1954)	AMINOP	1000 ug/kg 240 ug/kg	IP SC	Single Daily	Non-lethal Lethal
Small (1959)	AMINOP	480 ug/kg	IM	Daily for 8 days	Mild
WynnWilliams (1961)	AMINOP	300 ug/kg	SC	Daily	Lethal
Millington (1962)	AMINOP	600-2500 ug/kg 5000 ug/kg	IM IM	Single Single	Sub LD50 (LD50-3150 ug/kg, Lewis 1952) Lethal
Robinson (1966)	MTX	6 mg/kg 40 mg/kg	Oral	Single	Sub-lethal (LD50 60 mg/kg)
Loehry (1969)	MTX	7.5 mg/kg	IM	Single	Non-lethal
Altmann (1974)	MTX	5 mg/kg	SC	Daily for 3 days	Lethal (Robinson 1966, Ferguson 1950)
Taminian (1980)	MTX	30 mg/kg	IV	Single	LD50 (Chabner 1973)

MTX - Methotrexate
AMINOP - Aminopterin

Table 1.2 Studies of the effects of methotrexate and aminopterin on rat intestine.

evident in the stomach (Ferguson et al 1950, Small et al 1959, WynnWilliams 1961). Major changes were consistantly seen in the small intestine, specifically the jejunum and ileum. Woll and Olson (1951) stated that gastrointestinal lesions consistantly involved the small intestine first, then the colon and oesophagus, whilst the stomach escaped the process entirely. The microscopic changes seen in the small intestine of the rat are a consequence of the death of dividing crypt cells and the subsequent failure of new epithelial cells to migrate from the depths of the crypts up the walls of the villi (Millington et al 1962a, Margolis, Philips and Sternberg 1971). In an animal given a lethal dose, regeneration of the epithelial lining will not occur (unless the animal dies from the effects of the drug on another body system some days later). The atrophy of the epithelial lining leads to anorexia, ulceration, dehydration and death. The details of the mechanism of cell death have been the subject of speculation. Millington et al (1962a) felt that death occurred secondary to bacterial invasion, the bacteria gaining entry to the lamina propria and the blood vessels through breaches in the epithelium. Robinson et al (1966) felt that the loss of epithelial integrity allowed sodium ions and water to leak into the lumen and cause the animal to die as a consequence of dehydration.

Sublethal doses of methotrexate cause less devastating changes in the epithelium. Two phases can be distinguished microscopically. Initially, a degenerative period occurs which can last as long as 4 days. This is followed by a recovery period lasting in the order of 3 to 4 days, during which the villus anatomy is reconstituted (Millington et al 1962a). This sequence of events is in keeping with the action of methotrexate on the mitotically active cells of the crypts of Lieberkuhn.

Leblond and Stevens (1948) calculated that it takes 1.4 days for newly formed cells to migrate to the tip of the villus and be sloughed off. It has been shown by Margolis et al (1971) that a single dose of methotrexate stops mitoses in crypt epithelial cells with 6 hours of administration. Recovery corresponds with the fall in methotrexate levels below the threshold of $5x10^{-9}M$ as described by Chabner and Young (1973). The data of Taminiau et al (1980) suggests that mitotic recovery and cellular migration is accelerated above normal levels during the proliferative phase. They were able to detect labelled thymidine on the tips of villi, less than 12 hours after its injection into the animal. This represented a migration speed of 35 um/hour (normal 6-12 um/hour).

Vitale et al (1954) administered a fatal dose of aminopterin by daily injection. They felt that small intestinal mitotic activity could be seen to be decreased as early as 4 hours post injection. Mitotic activity was absent after 6 hours. They drew attention to the presence of inflammatory cell numbers which had greatly increased in the lamina propia of morbidly ill rats. In this study intestinal epithelium became thin and low, cuboidal cells replacing the normal columnar absorbtive cells. Cystic degeneration in the lamina propria occurred, and later, the epithelium was lost altogether. The mucous membrane was replaced by areas of haemorrhage and inflammation. Philips and Thiersch (1949) remarked on the inflammatory elements seen in their animals. The villi and submucosa had become infiltrated with neutrophils, lymphocytes and polymorphs by one to two days post injection. Accompanying the leucocytic infiltration was oedema and hyperaemia of the lamina propria and an extensive shortening and broadening of the villi.

WynnWilliams (1961) using a regime of daily subcutaneous injections (300ug/kg of aminopterin) noted similar degenerative changes. Villi became progressively stunted and atrophic. Some epithelial cells showed hydropic degeneration and crypt cells demonstrated mitotic arrest. Although epithelial cells became thin and atrophic in appearance, the continuity of the epithelium was maintained.

Millington et al (1962a) also noted extensive changes in the appearance of gut epithelium 2 days into their experiment. The epithelial cells had been reduced to a single squamous-shaped layer and these cells were being shed from the tips of the villi. The villi were reduced in height and the numbers of epithelial cells were greatly reduced. This tissue was able to recover. Crypt cells had regained their characteristic shape by day 4 and the villi had regained normal height by day 5. The recovery was complete by the seventh day.

Loerhy and Creamer (1969) administered 7.5 mg/kg of methotrexate to rats in a single intramuscular injection. They were able to observe a period of reduced cell production in the intestinal mucosa lasting for up to 3 days. This resulted in short hypoplastic villi. The mucosa was seen to regenerate over the succeeding 3 days and was of normal appearance by day 7. Considering these observations, it is remarkable that Robinson et al (1966) found no pronounced histological changes in their rats exposed to 6 mg/kg orally. In a second group of animals which received 40 mg/kg of drug, dramatic changes were seen. As early as the first day, vasodilation and oedema of the villi was observed. At this time villi had been reduced in height and mitotic activity ceased. By 48 hours, degeneration of the epithelial cells was obvious and cystic degeneration was seen in the crypts. By 96 hours, the villus epithelium

had largely disappeared, but mitoses were again occuring in the crypts. After a further 4 days, the histological appearance was reported to have returned to normal.

Altmann (1974) delivered a potentially fatal dose of methotrexate of 5 mg/kg daily, but only observed the rats for a maximum of 3 days. The earliest changes noted consisted of a decrease in crypt depth and a reduction in mitotic activity. There was a progressive decrease in villus and crypt area during treatment, culminating in the beginnings of epithelial denudation of the ileum by day 3. The remaining epithelial cells were seen to be cuboidal and flattened.

Taminiau et al (1980) observed microscopic changes from 24 hours and quantified a statistically significant reduction in villus height at this time. The villus cell numbers had also decreased by 24 hours and had not recovered by 96 hours. Whilst the number of mitotic figures was significantly reduced at 24 and 48 hours, by 4 and 8 days the number of mitoses was greater than that seen in normal rat small intestine.

PRINCIPLES OF CHEMOTHERAPY

While the scheduling of some cytotoxic agents is still based on empiricism, more predictable results can folllow the study of tumor cell kinetics. An understanding of the cell cycle is fundamental to the appreciation of this topic.

The Cell Cycle

The cell cycle proceeds in several phases following mitosis. Interphase is the period when the cell is not undergoing mitosis, but it

is still a dynamic period. It is divided in to 3 subperiods, namely G1, S-phase and G2.

During G1 DNA synthesis does not occur, but protein and RNA synthesis is very active. The duration of this phase is most variable and may be prolonged into a resting phase. Events in G1 have a significant bearing on the rate of cell replication and on whether or not the cell will differentiate into a specific phenotype.

S-phase is the period during which DNA is synthesized and the chromosomal component is duplicated. Concommitantly, RNA synthesis occurs as well as the synthesis of proteins which initiate DNA polymerase. The S-phase lasts 6-8 hours in rodent cells (Louis 1978) and the occurence of DNA synthesis can be confirmed by measuring the uptake of tritiated thymidine.

The G2 phase has less well defined biochemical functions although protein and RNA continue to be synthesized and the microtubules of the mitotic spindle are polymerized.

Mitosis occurs in M-phase and lasts approximately 0.5 to 2 hours in normal tissues of man and rats. In neoplasms, the M-phase varies in duration. Bizarre and grossly abnormal mitoses may result in cell death and this is an important pathway for cell loss (Louis 1978).

Chemotherapeutic agents act by interfering with either the synthesis or the function of nucleotides, nucleic acids, proteins or the mitotic spindle.

Tumor Cell Kinetics

Despite many years of intensive investigation, little progress has been made in identifying differences between normal and cancer cells which can be exploited in killing the malignant cells without harm to the normal cells. Differences have been determined in the biochemistry, surface properties, chromosomes and cellular kinetics, but none of these is unique to malignant cells or common to all malignancies (Clarysse, Kenis and Mathe 1976). The only chemotherapeutic agent which has a cytocidal action on malignant cells because they are malignant, is L-asparaginase. All others exert effects on normal cells (Mathe 1969, Oettgen et al 1967). Certain tumours lose the ability to synthesize asparagine (an amino acid which is non-essential for normal cells but essential for malignant cells). Tumour cells which are dependent on an exogenous supply of this amino acid, are rendered nonviable if asparagine is destroyed by asparaginase. For other drugs, chemotherapy is based on a difference in response between the normal cell and the malignant cell. The price paid for the similarity between the two types of cells is the drug toxicity.

A surface property of great interest is the loss of contact inhibition by malignant cells (Emmelot 1973). Unrestrained growth of malignant cells is likely to be explainable by differences in chromosomes. These may be secondary to an accumulation of somatic mutations or, the insertion of viral genome into the host chromosome.

A single malignant cell has the potential to divide and grow to a lethal number of cells and eventually kill the host. This was confirmed by Firth and Kahn (1937) when they were able to demonstrate that a

single leukaemic cell could give rise to a fatal burden of leukaemic cells in mice. The aetiology of solid tumours such as squamous carcinomas is likely to be more complex. In such cases a field of normal cells are usually transformed rather than a single cell. The progress from a small to a massive cell population is influenced by the growth characteristics of the tumour cells and by the behaviour of the host tissue. In fact, cancer cells do not necessarily divide more rapidly than the normal cells of the parent tissue. Bresciani (1968) found that cancer cells may not even replicate as quickly as the speed of the non-cancer cell line. The cell cycle time of a tumour is very often lower than that of normal tissue, such as bone marrow cells or gastrointestinal crypt cells. There is however, a difference between the growth fraction of cells in tumour masses and normal cells. That is, a greater proportion of cells in a malignant cell population are undifferentiated and capable of undergoing mitosis. This accounts for increased growth. Associated with this, we find a deficiency in the ability of the cells to differentiate and an enhanced ability to migrate and remain viable (Prescott 1972).

Malignant cells are not always in a reproductive cycle. Some cells are in a resting phase, G0 (DeVita 1971). Little is known about the differences between cells in G0 and G1, and the control of their conversion into a proliferative state. The cells may represent a population with a very long G1 period, rather than a different class of cells. Because they are not sensitive to cell cycle specific drugs, these cells may act as a source of malignant cells responsible for relapse after chemotherapy and also explain the appearance of metastases, years after the primary tumour has been controlled (Sugarbaker, Ketcham and Cohen 1971).

A tumour mass can be thought of as consisting of four types of cells (Fig. 1.4). These are:

(1) Dividing malignant cells

(2) Resting temporarily non-dividing malignant cells (GO)

(3) Differentiated cells

(4) Dying cells

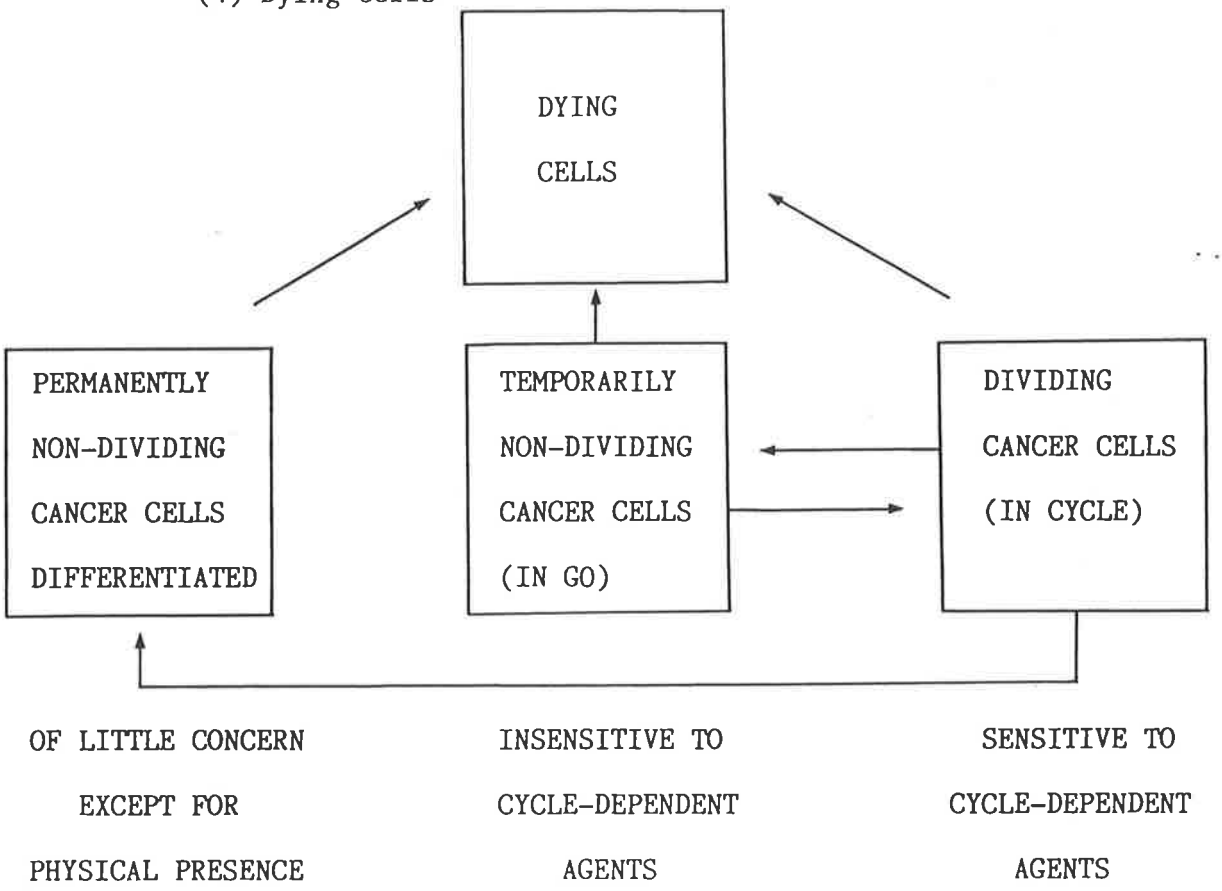


Fig. 1.4 Cell types in a tumour cell mass.
From: Clarysse, Kenis and Mathe (1976)

The non-maliganant cells are not the target of chemotherapy but, they may contribute to a large proportion of the tumour mass. The presence of these cells makes the interpretation of the effects of chemotherapy, in relation to changes in tumour diameter or volume, potentially invalid, as the direct effect on the malignant cell fraction is obscured. The clinical evaluation may not reflect the proliferative activity of a

particular malignant neoplasm as it does not consider cell loss. The cell loss factor varies very widely and can approach 100% (Valeriote and Edelstein 1977). Cell loss is contributed to by immune reaction, exfoliation, migration, old age, cellular defects or environmental factors, such as lack of blood supply. A tumour with a slow doubling time can still have a high growth fraction if it has the property of extensive cell loss. The relative populations of the cell types can also reflect local factors such as vascularity of the site. Tannock (1968) found that in mouse mammary tumour, the activity of malignant cells reflected the proximity of nutritive blood vessels. He was able to conclude that as a tumour mass enlarges, the growth fraction of the cells decreases. This is because the distance from blood vessels increases and blood supply is outstripped by tumour growth.

Both normal and neoplastic cells may be influenced by certain growth factors. Both appear to divide more rapidly when the cell population is small and more slowly when it is large. Early growth is clearly exponential, with a high growth fraction and short doubling times. As time passes, the doubling time lengthens and the growth fraction decreases. The mathematical model relating to this phenomenon was described by Gompertz, an eighteenth century mathematician (Laird 1964) (Fig. 1.5). The implication of this model is that while the tumour size is small, cell dynamics are favourable for cell-cycle dependent drugs to be effective. Solid tumours are generally diagnosed late in the course of the disease. At this stage they have advanced into the plateau phase of the growth curve, which makes them less amenable to this style of therapy. In practice, clinical responses of the malignancies do not always fit predictions based on kinetic considerations. This fact underlies the inadequacies of our knowledge of this subject (Hall 1971).

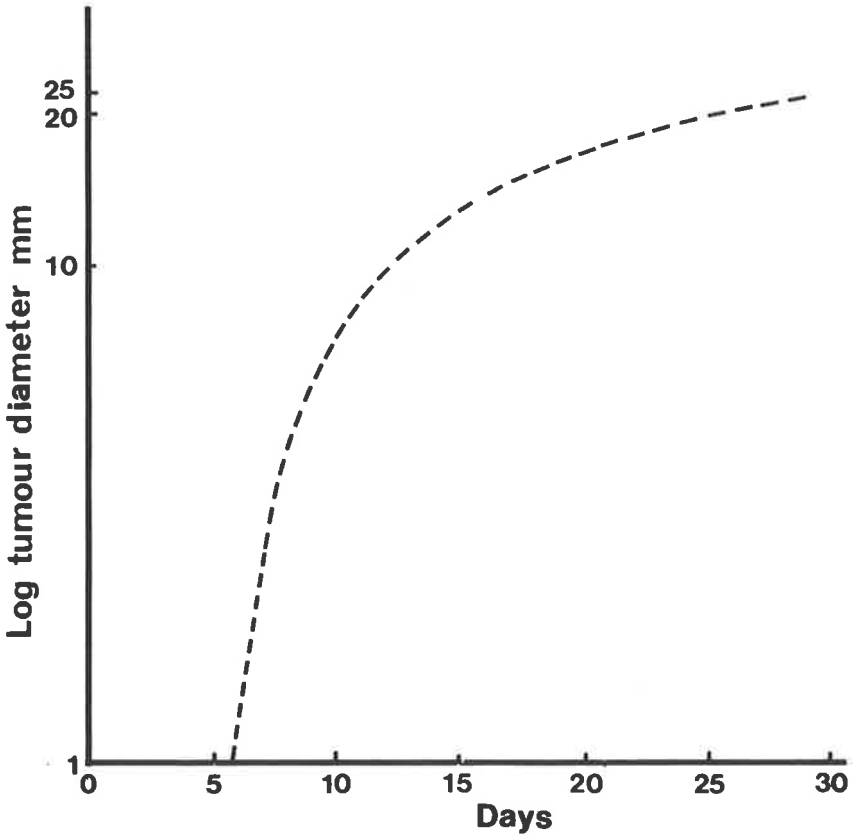


Fig.1.5 Tumour growth following s.c. injection of 10^4 L1210 leukaemia cells in mice. Growth follows a Gompertz curve. From Clarysse, Kenis and Mathe (1976).

A number of principles govern the use of cytotoxic agents. Some of these were elucidated by Skipper, Schabel and Wilcox (1964, 1965) using transplantable L1210 leukaemia in mice. The most basic is that a single malignant cell has the potential to multiply until the malignant cell burden kills the animal. The implication for chemotherapy is that the therapy should be designed to kill all of the malignant cells if a cure is to be guaranteed.

Cytotoxic drugs kill malignant cells in a logarithmic fashion. A given dose kills a certain proportion of the malignant cells and not a certain number. This occurs regardless of the number of cells present at the commencement of therapy. The fraction killed is never 100%. A drug dose which reduces the cell number by 99% will reduce any population of similar cells by two orders of magnitude. It has a 99% chance of eradicating the cancer if only one cell is present. There is an inverse relationship between cell number and curability by chemotherapy. This fact tends to support the use of chemotherapy as an adjunct to other therapies in the treatment of solid tumours, where large numbers of cells are present. A single malignant cell mass 1 mm in diameter may contain as many as 10^6 cells and a mass of diameter 1 cm may contain 10^9 viable cells (Schabel 1975b). Cells surviving an initial dose will resume proliferation at the rate seen before chemotherapy (Skipper 1968). This means that delay between successive doses of drug may see the malignant cell population re-established. A potentially more serious possibility is that cells within solid tumours which have progressed to the plateau phase of growth may re-enter the exponential growth phase, owing to a decrease in the total population (Schabel 1969). Challenging the remaining cell population with a further dose of drug has an effect which depends on the number of cells which survived and the interval

between the two treatments. It is also influenced by the doubling time of the tumour and the possibility of the selection of resistant cells and their subsequent proliferation.

The schedule of drug administration is also important in determining the effect of a course of chemotherapy. The same dose of a cytotoxic drug will not produce the same result if the schedule of administration is changed. The time chosen between two drug administrations must be reduced to the minimum which allows normal cell populations to recover. In the human situation this time interval is generally 28 days in the case of drugs whose primary toxicity is to bone marrow (DeVita 1971, Djerassi 1975).

Chemotherapy is more likely to be curative if it is directed against a small malignant cell population. In large tumours several factors make success less likely. The growth characteristics of the tumour cells may be reduced owing to hypoxia. Drug penetration may be difficult owing to poor vascularity. The larger the malignant cell population the more likely is the presence of lines of resistant cells.

In order to optimally schedule chemotherapy, there are a number of factors which ideally should be known:

(1) The duration of the cell cycle,
(2) The number of malignant cells present,
(3) The rate of cells entering S-phase,
(4) The rate of recovery of host cells,
(5) Whether the chemotherapy will enhance tumour or normal cell proliferation,

(6) The minimum cytotoxic level, and

(7) The rate at which drug disappears from body fluids.

The cell cycle characteristics of the tumour may influence the type of drug chosen as well as its scheduling. Agents can be classified on this basis into two types as determined by Bruce, Meeker and Valeriote (1966). Cycle dependent drugs effect cells in a proliferative phase of the cell cycle but do not damage resting cells. The toxicity of cycle dependent drugs is determined by their concentration. Therefore, they can be very effective if administered intermittantly at the highest dose which will not cause undue toxicity (Mathe, Schreider and Schwarzenberg 1970). Phase dependent drugs effect cells in a particular phase of the cell cycle, G1, S, G2 or M. Unless different mechanisms of cell damage operate at higher drug levels, increasing the dose of these agents above an optimum does not kill more cells. Phase dependent agents will exert an increasingly toxic effect the longer a critical concentration is maintained, as more cells are able to enter the sensitive phase of the cell cycle. When administered over a short time period, phase dependent drugs tend not to be highly toxic even at high dosage (Kenis et al 1971). Phase specific agents can therefore be administered in fractionated schedules or as infusions, unless they have a long half-life. Methotrexate lends itself well to this type of approach.

Drug Resistance

Resistance of micro-organisms to a drug is a well known phenomenon. Resistance of cancer cells to the cytotoxic effects of an antineoplastic drug is an equally serious problem. When resistance is possessed by normal cells, but is not a feature of the malignant cell population, one

can expect that the cancer cells will be killed preferentially. In a malignant cell population resistance may be inherent, or acquired. The former situation is most likely explained by the low growth fraction and S-phase population in the neoplasm. Some cells may lack an efficient membrane transport system for methotrexate and thus remain impervious to high sustained extracellular drug levels (Fischer 1962). Alternatively, it has been postulated that some cells have a capacity to rapidly turnover dihydrofolate reductase (Hryniuk and Bertino 1969) and are able to provide a high intracellular level of the enzyme (Kufe, Wick and Abelson 1980). An increased resistance also becomes manifest when a malignant lesion grows to a large size. The centre of such a lesion may not receive a high enough level, or duration of exposure to the drug, for it to be effective. This is described as a pharmacological sanctuary. Altered cellular kinetics can also occur in such an area, minimizing the effects of the drug.

Acquired resistance to antifolates can occur by two major mechanisms. The first of these is the development of the ability of cells to synthesise dihydrofolate reductase (Bertino et al 1962). This occurs by the process of gene amplification (Schimke 1980). Cells surviving an initial dose of methotrexate contain extra chromasomal genes for the manufacture of high levels of dihydrofolate reductase. The second way in which drug resistance to methotrexate may be acquired is for the transport of methotrexate to become less efficient.

Cytotoxic drug resistance is a problem seen on the re-administration of a drug because the resistant cells have been selected for by the initial drug dose. These cells can act as progenitors for a whole new line of resistant cells (DeVita 1983). Whilst malignant cells

are able to develop resistance, normal cells do not appear to have this ability. Gastrointestinal epithelium and bone marrow cells are succeptible to drugs such as methotrexate and remain so. This phenomenon has never been fully explained (DeVita 1983).

Resistance can be partly overcome by an increase in the dosage of methotrexate and also by the use of larger doses at intervals rather than small intermittant doses over a long period (Hill et al 1979).

HIGH DOSE METHOTREXATE

The problem of resistant cell populations has been addressed by the introduction of high doses of methotrexate. Two factors make the use of methotrexate in high doses, without producing severe toxicity, a possibility. The first is the availability of agents such as leucovorin to rescue normal cells from the effects of the drug. The second is the fact that toxicity to methotrexate is a function of the time over which DNA synthesis is arrested rather than of the total dose of the drug. As long as the intracellular levels of methotrexate decrease sufficiently to allow dissociation from dihydrofolate reductase before the time threshold for normal cell damage has expired, the peak levels of drug achieved do not greatly contribute to further toxicity.

There are a number of theoretical grounds which support the use of high doses of methotrexate for solid tumours. These relate both to the resistance of the cell mass in in vivo conditions and to the resistance properties of individual cells.

A factor militating against the action of methotrexate when directed against solid tumours is the difficulty in delivering a sufficient concentration of drug into the cells of the tumour mass. As indicated earlier, as the distance from the blood supply increases the availability of the drug decreases. However, higher doses of drug have the potential to reach the pharmacological sanctuaries in the centre of the mass of cells as a consequence of a more favourable concentration gradient. If sufficient intracellular levels of methotrexate can be established they may be maintained for an extended time. This is because diffusion of the drug out of the tumour may also be decreased as the blood drug level falls. Comparatively, loss of the drug from normal cells would be rapid. This situation is also favourable for the selective rescue of normal cells by leucovorin. The rescue agent can rapidly enter normal cells, whilst in the malignant cells, methotrexate continues its action as more tumour cells enter S-phase (Goldman 1981).

White (1981) reviewed the biochemical relationship between methotrexate and dihydrofolate reductase. It has been assumed that because methotrexate has a high affinity for binding with its target enzyme, recovery of a cell from methotrexate exposure would be slow, being achieved primarily by the synthesis of more stocks of enzyme. It is now known that if intracellular methotrexate levels are reduced to a level where no free methotrexate is left, tetrafolate synthesis will resume. In fact, free intracellular levels of methotrexate must be maintained at a level of up to four orders of magnitude above the Kl to ensure total tetrafolate synthesis inhibition (White and Goldman 1976).

Methotrexate actually acts as a rapidly reversible low affinity inhibitor of its target enzyme. A very low level of free dihydrofolate

reductase is sufficient to meet cellular demand for tetrafolate. The requirement for free intracellular methotrexate must be provided from extracellular supplies, which in turn must be maintained at a level of perhaps an order of magnitude higher than intracellular levels (Goldman et al 1976).

Tumour cells which are resistant to methotrexate may have one or more properties which work against the saturation of dihydrofolate reductase. Acquired resistance may involve a decreased ability to uptake the drug. Such cells may possess a poor active transport mechansim for methotrexate (Fischer 1962, Djerassi et al 1967, Niethammer and Jackson 1975). At low concentrations methotrexate uses the active transport mechansim to enter cells. In high doses a passive non-energy dependent process operates (Bender 1975). Such a process is favoured by the concentration gradient achieved by high serum drug levels.

Resistant cells may have greater than usual stock of dihydrofolate reductase (Fischer 1961). Some cells can develop higher levels of the enzyme during therapy. This observation correlates well with the clinical finding that the most dramatic tumour shrinking effects usually occur during the early stages of therapy. Other cells may have a weaker binding between the enzyme and methotrexate (Jackson, Hart and Harrap 1976). Higher doses of methotrexate also have the potential to overcome these problems.

Again, transport resistance in tumour cells may improve the therapeutic advantage of methotrexate when leucovorin rescue is used. As leucovorin is transported by the same transport system as methotrexate,

the resistant cells may exclude the low dose leucovorin, while normal cells take it up and are rescued.

Goldman (1977) reviewed the possible mechanism for the destruction of tumour cells which are not in S-phase. He stressed that such mechanisms could only operate under very high doses of drug, but that anectdotal evidence for their existance is seen when high-dose methotrexate protocols inhibit slow-growing solid tumours. Non-proliferating cells continue to incorporate tritiated deoxyuridine and require tetrahydrofolate. This has been attributed to mitochondrial DNA synthesis or DNA repair. The lethal effect of MTX could then be due to the inhibition of DNA repair or to the depletion of tetrahydrofolate co-factors required for RNA synthesis or the loss of short lived proteins. The importance of these mechanism in vivo is not known.

METHOTREXATE IN THE TREATMENT OF HEAD AND NECK CARCINOMA

The value of methotrexate in the treatment of advanced squamous cell carcinoma of the head and neck was first explored in the 1960's. Huesby and Downing (1962) were perhaps the first to demonstrate that some head and neck carcinomas are responsive to methotrexate. Following this initial attempt various routes, doses and schedules were used with or without the help of rescue agents. Curative results have remained rare. In many cases a decrease in tumour size occurred and in some, clinically obvious tumour disappeared. Unfortunately, relapse generally supervened in these cases after a short period. Mead and Jacobs (1982) reviewed the results of a number of representative studies using methotrexate in various schedules. The results of this review confirms this bleak prognosis (Tables 1.2 and 1.3).

Table 1.2 Methotrexate Therapy in Head and Neck Cancer

Author	Drug Schedule		Evaluable Patients	Response*		MDR
				CR	CR+PR	
Papac et al (1963)	(1) 15 mg/day x IV	4 weekly	23	0	17	1
	(2) 5 mg/day x 5 PO	4 weekly	24	0	17	
Lane et al (1968)	20-50 mg IV	every 4-7 days	27	15	52	3
Leone et al (1968)	(1) 60 mg/m^2 IV	weekly	30	26	57	4.5
	(2) 40 mg/m^2 IV	twice each week	5			
Papac et al (1978)	0.8 mg/kg IV/IM	twice each week	35	0	20	4
Priestman (1973)	15 mg PO	weekly	40	0	8	7

MDR = Median Duration Response (mo)
CR = complete remission
PR = partial response (>50%)

Table 1.3 Moderate Methotrexate with Leucovorin Rescue in Head and Neck Cancer

Author	Drug Schedule		Evaluable Patients	Response*		MDR
				CR	CR+PR	
Levitt et al (1973)	0.24-1 g/m^2 IV over 36-42 hrs	every 2 weeks	25	-	60	2
Capizzi et al (1970)	0.24 g/m^2 IV over 24 hrs	every 4 days	21	-	62	4
Buechler et al (1979)	70 mg/kg IV	every 3 weeks	23	9	26	4
Frei et al (1980)	1.7-5 g/m^2 IV	weekly	60	-	50	2

Tables modified from Mead and Jacobs 1982.

It is clear that methotrexate is unlikely to be able to destroy all of the cells in a large mass of squamous carcinoma. However, this does not mean that the drug does not have a role in treating this type of neoplasm. Traditionally, chemotherapy for squamous cell carcinoma of the head and neck has been reserved for the treatment of lesions which are not amenable to either surgical eradication or treatment by irradiation. These lesions are the larger and less accessible masses. Methotrexate also has been used in cases where metastases have already occurred. Chemotherapy has also been reserved for those patients in whom a course of radiotherapy or an operation, has failed. The vast majority of stage III or IV lesions (Appendix III) subsequently treated by chemotherapy are not successfully controlled, resulting in three year survival rates ranging from 3% to 34% (DeConti 1981). This "last resort" approach to the use of methrotrexate and other drugs has produced a predictably poor response for several reasons. The patients are generally elderly and delibitated by their malignant disease and other diseases, such as vascular and respiratory disease and alcoholism. Such patients tolerate cytotoxic therapy poorly and their malignant disease responds poorly to it (Amer, Al-Sarraf and Vaitkevicius 1979). The mass of cells, or masses which the drug has to attack, are very large. Like radiotherapy and surgery, chemotherapy is less effective against a large number of cells. Poor blood flow in such a mass makes it unlikely that all of the cells will receive a lethal drug dose. Furthermore, a large mass of cells is more likely to contain a number of resistant cells. A course of chemotherapy may effectively select for these cells which survive and then have the potential to proliferate. The cell lines remaining will fail to be affected by a second challenge by the same drug. Where other treatments have preceeded the chemotherapy, scarring and fibrosis occur in the area. The resultant poor blood supply in this situation limits

the effectiveness of the chemotherapeutic agent. For example, Sealy and Helman (1972) compared the efficacy of intra-arterial methotrexate between patients who had previously been treated with radiotherapy and a similar group who had not. Forty four percent of the former group responded compared with 84% of the previously untreated group.

The effectiveness of a drug used against squamous carcinoma of the head and neck is not usually assessed in terms of a cure rate. Instead, response rates have generally been recorded (Appendix II). A complete response does not denote a cure, but rather a reaction to the drug. One can expect a recurrence of the neoplasm in most of these cases. When used against advanced (stage III and IV) disease, standard-dose methotrexate (without rescue) can be expected to produce a response rate of approximately 29% (Pitman and Bertino 1984).

Such discouragement has led some investigators to examine the benefits of using cytotoxic drugs as the initial therapy for head and neck carcinomas. This approach has certain theoretical and practical advantages. Pain associated with the primary lesion is reduced following the chemotherapy (Desprez et al 1970). Some tumours initially considered inoperable, have become operable (Shapshay et al 1978). Small satellites of malignant cells around the main mass may be destroyed and micro-metastases about the body may be eliminated by the chemotherapy. The risk of dissemination of viable malignant cells during subsequent surgical manipulation of the neoplasm may be reduced.

Pre-operative chemotherapy of head and neck carcinomas with methotrexate was pioneered in the 1960's when the intra-arterial route was used (Desprez et al 1964, 1966, 1970). Later, intravenous

administration (Tarplay et al 1975) was employed. These early studies demonstrated sometimes spectacular clinical effects, with disappearance of viable malignant cells being evident on microscopic examination of some of the respected specimens. Importantly, the oncologist was able to assess the response of the neoplasm in terms of histological changes, rather than having to rely on the nonspecific clinical response criteria. Some insights into the cellular effects of the drug in vivo was gained.

Pre-operative chemotherapy regimes are now in widespread use in the treatment of head and neck carcinomas. These have employed single agents and more recently, multiple cytotoxic drugs. A number of pilot studies are now available which evaluate the results of this approach (Salmon and Jones 1981). The data emerging suggests that advanced squamous cell carcinoma of the head and neck can be effectively debulked with chemotherapy delivered before surgery or radiotherapy. Response rates tend to be significantly improved compared with those seen after prior surgery or radiotherapy has been given. One must await the results of prospective, randomized clinical trials before the ultimate effect on cure rates can be assessed (Mead and Jacobs 1982). Only the ability to achieve a complete response of lasting duration would mark true progress in the treatment of this disease.

CHEMOTHERAPY OF SOLID MALIGNANT NEOPLASMS IN ANIMALS

Whilst the efficacy of methotrexate in eradicating leukaemic cells in rodents has been well established (Goldin et al 1959), the value of the drug in the eradication of solid rodent lesions is variable. Skipper and Schmidt (1962) presented data describing the results of confronting

various rodent neoplasms with a variety of drugs including methotrexate. Methotrexate delivered in divided intraperitoneal doses at LD10 level failed to inhibit the growth of subcutaneous carcinoma 775 to 40% of that of controls. This regime also failed against Walker 256 carcinoma. However, the drug did exhibit effectiveness by eradicating Dunning leukaemia when given in a similar regime.

Desprez et al (1970) carried out experiments on induced carcinomas in rodents in conjunction with their clinical studies. They subjected rodent DMBA induced breast adenocarcinoma and Walker sarcoma to methotrexate without noting a significant clinical response. They concluded that because the response of an experimental neoplasm to methotrexate cannot be anticipated, such lesions should be challenged with the greatest dose possible. This would improve the likelihood of a significant response occuring.

Franchi, Moretti and Garattini (1970) subjected rats suffering from a transplantable rhabdomyosarcoma to methotrexate. The drug was delivered daily by intraperitoneal injection for 7 days and then on alternate days until 19 days had passed. The dose given was considered to represent 1/6 of the LD 50 (measured over 6 days). The weight of the sarcomas did not change significantly in this study.

Sindran et al (1974) used the same malignant neoplasm but administered the drug via intra-arterial infusion. Lesions were implanted in each ear and the drug was administered via a catheterized common carotid artery. Three dose schedules were employed. A continuous 7 day infusion of 3 doses up to a maximum of 0.28 mg/kg/day, produced significant reduction in lesion size at each dose on the infusion side,

but only at the highest dose level on the contralateral side. This was interpreted as demonstrating that intra-arterial infusion is superior to systemic administration in such a model. An infusion of methotrexate over days one and 4, for a total dose of 1.0 mg/kg, was also given. This method produced significant tumour regression while causing less toxic effects than the 7 day schedule.

A third group of rats received 7 days of continuous infusion plus citrovorum factor intraperitoneally, 6 hourly. Lesion shrinkage in this group was less than in the unrescued group, but the LD 50 was increased 2.5 times. This result is due to the malignant cells being rescued along with the normal cells and was consistant with the results of Goldin et al (1955, 1966).

Schouwenburg, VanPutten and Snow (1980) described the results of another experiment, again delivering methotrexate by intra-arterial infusion. On this occasion, the target was a moderately differentiated skin squamous cell carcinoma. Seven days of constant infusion of up to 1.4 mg/kg/week produced no reduction in carcinoma growth attributable to the drug.

Falk et al (1982) studied the effects of 5 fluorouracil and methotrexate on two rat neoplasms after intradermal sensitization to these agents. The neoplasms were a chemically induced bladder carcinoma and a spontaneously arising mammary adenocarcinoma. Methotrexate was delivered weekly by pulse dose of 0.75 mg/kg intravenously. This produced no effects on lesion growth or animal survival, unless the animal had been previously sensitized against methotrexate by intradermal injection.

Drago and Palmer (1983) studied the value of methotrexate in inhibiting the Nb rat prostatic adenocarcinoma. In a dose of 0.5 mg/kg there was no significant decrease in tumour volume when compared to controls. At a single dose of 50 mg/kg the volume of the tumours was significantly reduced as were the number of metastases. This study lends support to the proposition that high dose regimes are more likely to be effective against solid neoplasms.

From these examples, it can be seen that a number of solid rodent neoplasms appear to be resistant to methotrexate and others are only likely to respond to higher doses or intermittent infusions. This is in keeping with tumour cell population kinetic principles, which predict the need for high dose intermittent drug delivery against solid tumours with a low growth fraction of cells.

CHAPTER 2

AIMS OF THE INVESTIGATION

Since 1983 a number of patients seen by the Oral and Maxillofacial Unit, Department of Dentistry, University of Adelaide and the Royal Adelaide Hospital, who have operable oral squamous cell carcinoma of T2 size or larger, have been admitted for a course of cytotoxic therapy prior to resection of the carcinoma. One form of chemotherapy has consisted of an infusion of methotrexate, followed by leucovorin rescue as recommended by Capizzi et al (1970) (Appendix IV). This therapy is aimed at reducing the local, regional and distant recurrence of the neoplasm and its metastases. To date the clinical results of this treatment have been variable. Some lesions have proven refractory to the therapy while others have responded to varying degrees, including clinical disappearance of the primary lesion. In some of the latter examples, microscopic examination of the surgical specimen has not revealed any viable malignant cells. Despite this observation, some of the same lesions have recurred locally or spread, either regionally or systemically.

Little experimental data is available regarding the microscopic effects of methotrexate-leucovorin regimes, in relation to their effects on oral carcinoma and normal tissues, such as gastrointestinal tract. Because such data is not easily obtained under controlled conditions in human studies, it was decided to explore aspects of these questions in an animal model.

Previous experiments in the Department of Dentistry have confirmed that oral squamous cell carcinoma can be consistently induced in laboratory animals (Appendix V). This model would allow investigation of the effects of methotrexate-leucovorin infusion on both oral neoplasms and normal tissues at the same time.

As has been discussed, induced or transplanted rodent malignant neoplasms are not necessarily responsive to cytotoxic drugs such as methotrexate and the toxicity of methotrexate may vary with the size and strain of the animal, the route of drug delivery and the drug regime used. Because of a lack of pre-existing data concerning the infusion of methotrexate to the particular animals used, it was felt that the study of methotrexate-leucovorin on induced rat neoplasms should be preceeded by the study of the toxicity of methotrexate infusions, with and without leucovorin, on these experimental animals and of their effects on normal rat tissues.

With a view to the eventual challenge of oral neoplasms with particular drug regimes, it was felt that the LD 10 level of methotrexate toxicity would be an appropriate trial therapeutic dose. A higher dose of methotrexate could be given with the same resultant toxicity, if leucovorin was additionally administered. This regime might have a greater change of affecting experimental carcinomas.

The small intestine is the most sensitive organ in the rodent to methotrexate. Therefore, this organ was chosen for the study of the histological consequences of therapeutically aimed regimes of methotrexate on a susceptible normal tissue. From such a study one would like to confirm the efficacy of the methotrexate regimes in inhibiting

mitotic activity for a significant period and observe the histological consequences of the inhibition. A non-lethal dose should then allow the observation of a period of recovery to the original morphology. We would also like to confirm the ability of leucovorin to protect the normal tissue from the toxic effects of a high dose of methotrexate and to compare these findings to those when methotrexate is used alone.

The principal objectives of this study were to:

1. develop an animal model allowing infusion of the chemotherapeutic agent methotrexate (with and without leucovorin rescue).
2. record the clinical toxicity criteria following various doses of the agent and to establish an LD 10 and LD 90 infusion regime.
3. observe and record the microscopic changes in the rat small intestinal mucosa following various methotrexate infusion regimes.

CHAPTER 3

MATERIALS AND METHODS

Experimental Animals

Male Porton strain albino rats were selected as the experimental animal. The rats were delivered at 12 weeks of age from the Central Animal House, University of Adelaide. On arrival at the Department of Dentistry the rats were housed in polypropylene cages, 3 animals occupying each cage, and fed standard pellets (Appendix VI) and tap water ad libitum. The animals were kept in the animal house of the Department of Dentistry, University of Adelaide for at least one month before experimentation was begun. At this time the average weight of the animals was 400 grams.

Experimental Drugs

Methotrexate was received as a solution of sodium methotrexate at a concentration of 25 mg/ml (David Bull Laboratories, Australia). The stock solution was subsequently diluted with normal saline to the appropriate dosage contained in 1 ml of solution, for the infusion phase of the study. This was administered by continuous intraperitoneal infusion over 24 hours preceeded by a loading dose consisting of 10% of the solution. An electric powered infusion pump (Paton Industries Pty. Ltd. Australia) (Fig. 3.1) was used to drive the plunger of a 1 ml. tuberculin syringe.

Leucovorin was received as a solution of leucovorin sodium at a concentration of 10 mg/ml. (David Bull Laboratories, Australia). Again

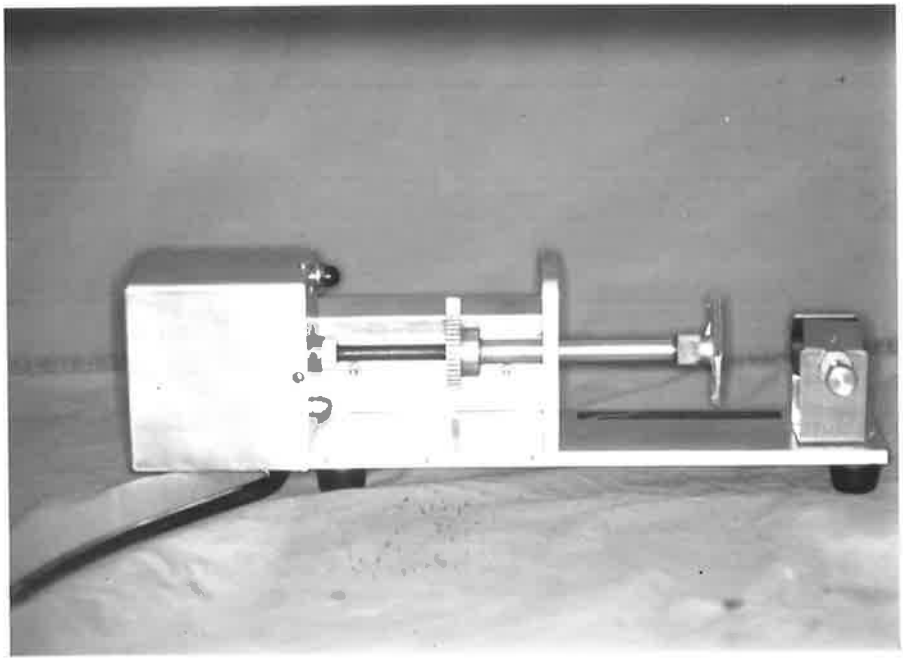


Figure 3.1: Infusion pump

this solution was diluted with normal saline as necessary. This drug was also administered by a continuous intraperitoneal infusion preceeded by a loading dose of 10% of the solution.

Anaesthesia

Animals were individually placed in an anaesthetic chamber (Fig. 3.2) and were lightly anaesthetized with a mixture of N_2O and Halothane (Fluothane^R) and oxygen. (If not further sedated, the rats recovered from the effects of these drugs within 2 to 3 minutes). The rats were then weighed and given an intraperitoneal injection of pentobarbitone sodium (Nebutal^R) at a dose of 6 mg/kg body weight. This was injected with minimal trauma in the motionless rat. The dose of anaesthetic was sufficient to provide over one hour of unconsciousness.

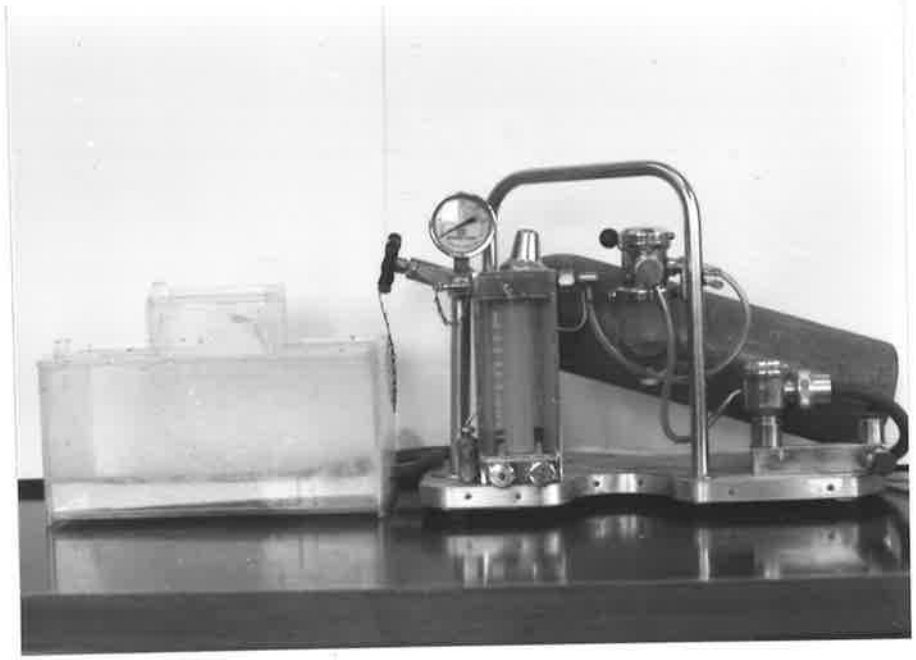


Figure 3.2: Anaesthetic machine and chamber.

Experimental apparatus

During the period of infusion the animals were individually housed in 25 cm lengths of PVC piping. The diameter of the pipe was 7.5 cm. One end of the pipe was left uncovered. The back end was capped by a PVC seal. A 1.5 cm hole was drilled through the cap to allow passage of the animals tail and the catheter. The PVC pipe was liberally fenestrated particularly on the floor, to allow the passage of excreta and for ventilation (Fig. 3.8). Animals were acclimatized to these pipes by placing pipe in the cage some weeks before the experiment. The catheters were made from a thin (0.8 mm) polyethylene tube which was attached to a 25 gauge needle and cut to length to contain 0.1 ml of liquid. The indwelling end was bent into a figure of eight configuration and secured by a single tie. This prevented its inadvertant dislodgement from the peritoneum while allowing it to be removed with minimal force (Fig. 3.3).

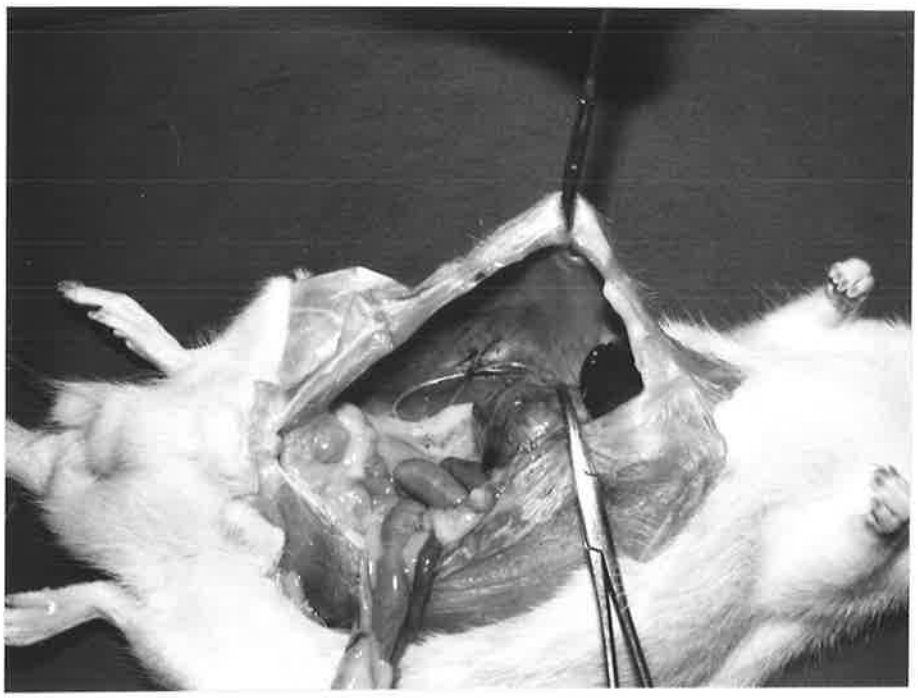


Figure 3.3: Autopsy view of catheter in situ, placed in peritoneal cavity. Figure of eight configuration secures it.

Operative technique

For insertion of the catheter, the anaesthetized animals were placed on their backs on an operating board (Fig. 3.4). A small incision was made in the right lateral abdominal skin to expose the abdominal muscles. A stab incision was used to breach the wall of the abdomen. A pair of mosquito forceps was passed subcutaneously from the skin incision posteriorly, past the groin to exit the skin adjacent to the scrotum (Fig. 3.5). The indwelling catheter was grasped in the forceps and pulled through the tunnel (Fig. 3.6). The tip of the catheter was passed into the peritoneal cavity, taking care to avoid traumatizing the adjacent intestine. Two catgut sutures were sufficient to securely close the deep wound and the skin was similarly closed. The catheter was necessarily placed subcutaneously to avoid the possibility of the animal dislodging it while undergoing infusion.

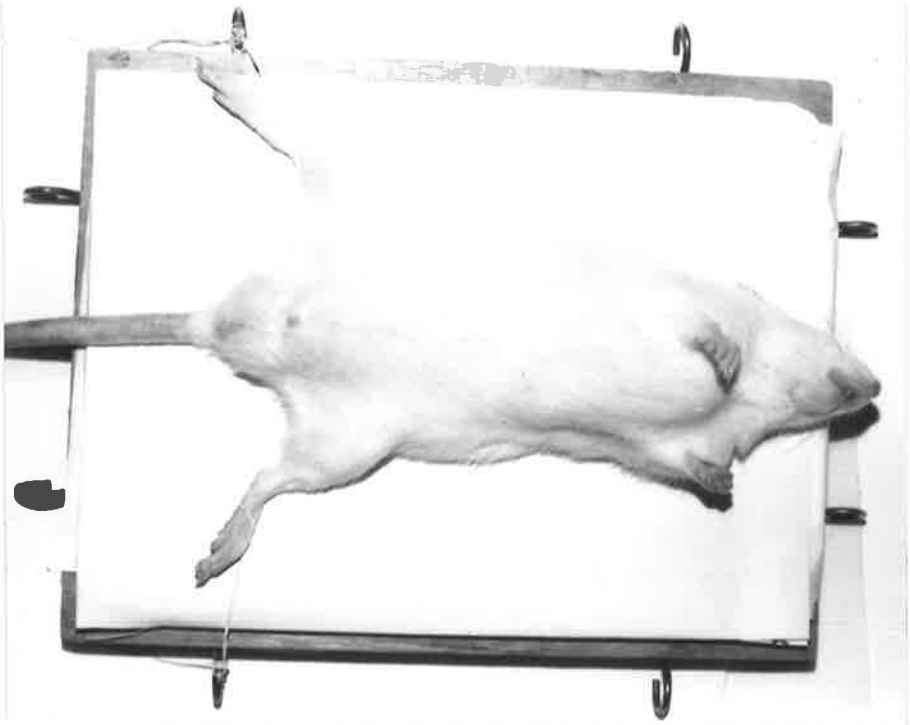


Figure 3.4: Anaesthetized animal in position on operating board.

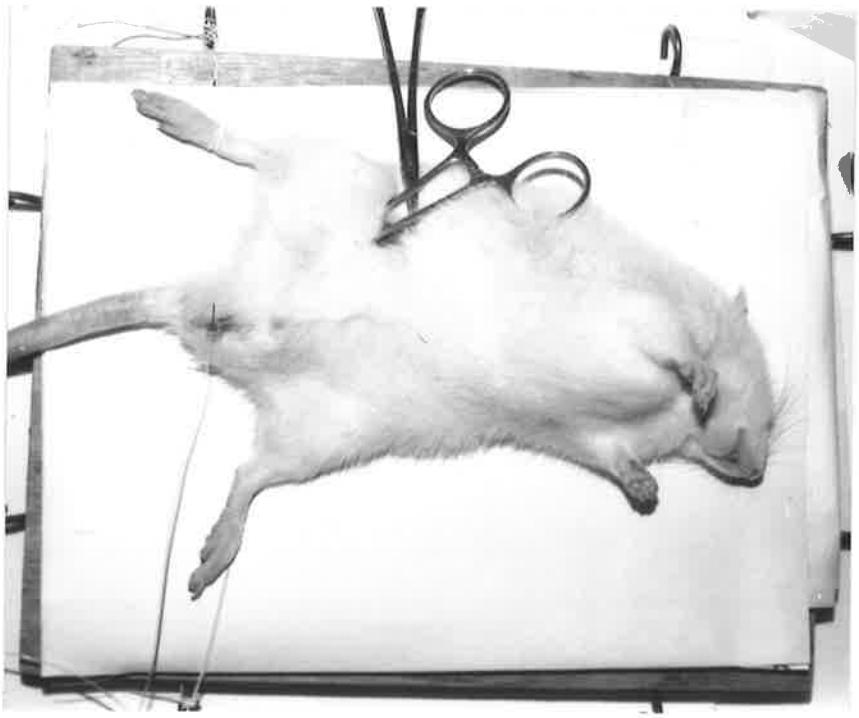


Figure 3.5: Mosquito forceps pulling catheter through subcutaneous tunnel.

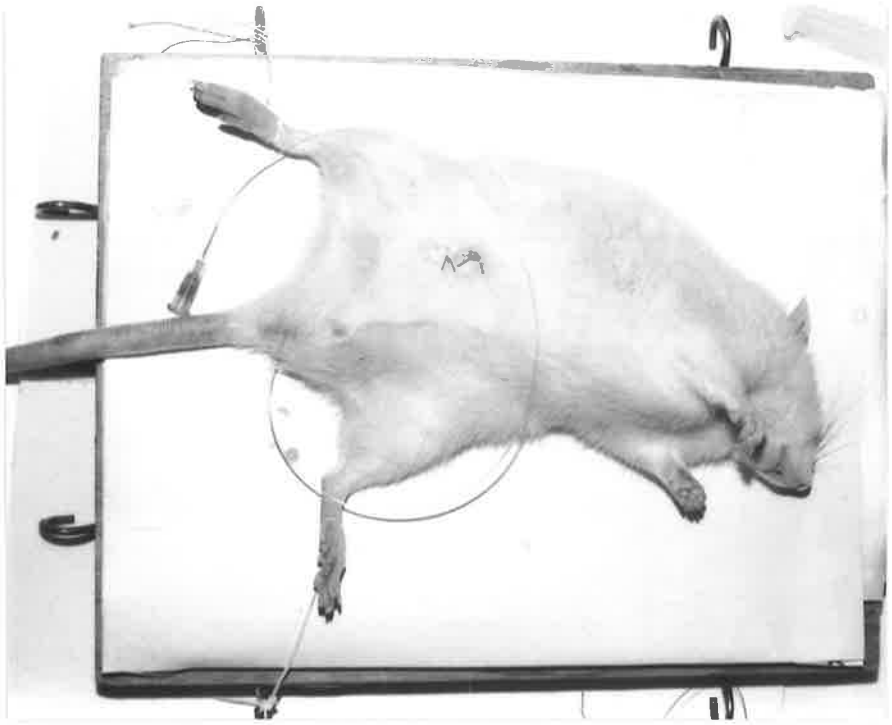


Figure 3.6: Cathether in situ, skin wound sutured.

Subsequent to placement of the catheter, the animals tail and the catheter were placed through a hole in the cap of the PVC piping. The rats tail was wrapped in adhesive tape and the animal placed inside the length of PVC piping (Fig. 3.7, 3.8). A water bottle was placed adjacent to each rat as was a container of food pellets. At the end of the infusion period each animal was freed by removing the adhesive tape. A gentle pull on the catheter was all that was necessary to dislodge it from the peritoneal cavity. After being weighed, the rat was returned to its cage for observation.

Monitoring of Animals

The animals were weighed on removal from the experimental apparatus and on each subsequent day up to a maximum of 20 days, or until the time of sacrifice. The behaviour and appearance of the animals was also noted on a daily basis. Note was made of the food and water intake, the condition of the fur and skin and the state of the stools. Any animal seen to be suffering from morbid toxicity prior to its scheduled time of sacrifice was immediately killed.

Figure 3.7: Animal positioned in PVC piping.

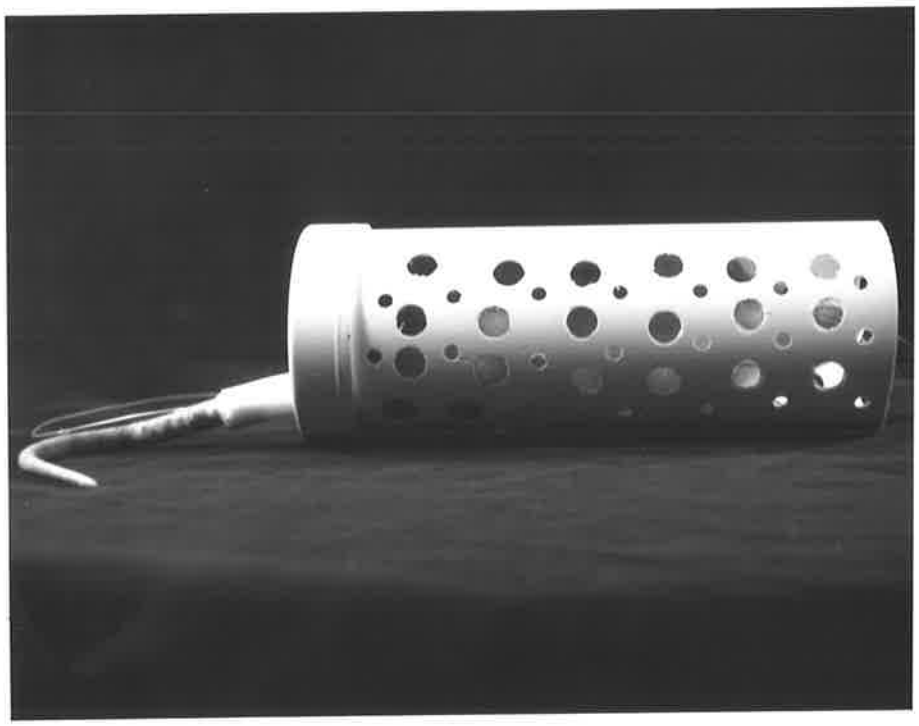


Figure 3.8: Lateral view of fenestrated PVC restraint used to house animals during infusion. The adhesive tape around the base of the animals tail secures the animal within the tubing.

Collection of Tissue

At the time of sacrifice, each animal was again weighed and examined to determine its physical condition. The animal was swiftly killed by cervical dislocation. A longitudinal midline incision was then made through the skin and abdominal muscle to expose the abdominal contents. The gastrointestinal tract was removed in one piece, from the cardiac sphincter to the rectum. The intestines were gently teased from the mesentery allowing them to be extended to their full length (Fig. 3.9). The complete specimen was carefully wound around a piece of

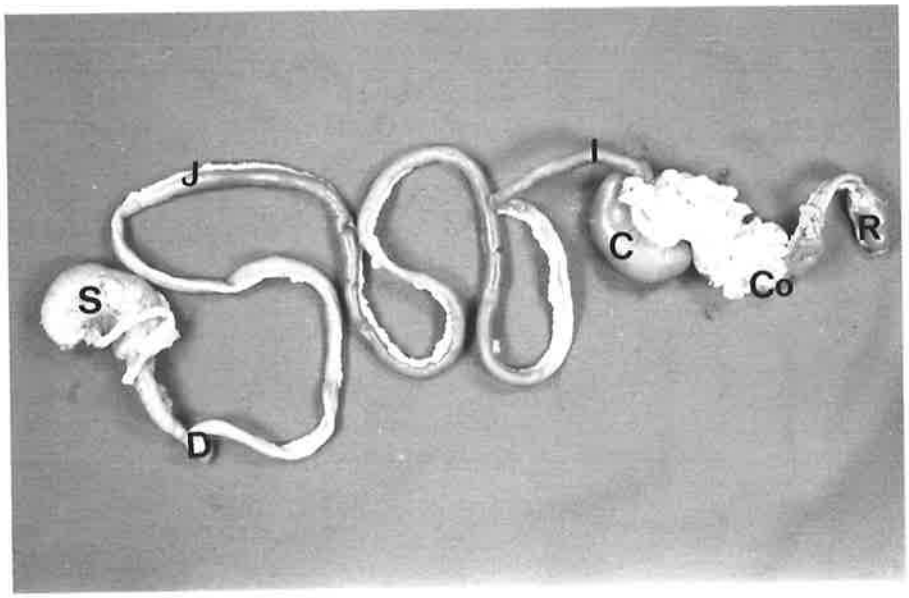


Figure 3.9: Fixed gastrointestinal specimen. Stomach (S), Duodenum (D), Jejunum (J), Ileum (I), Caecum (C), Colon (Co), Rectum (R).

cardboard and secured at each end. Ten per cent neutral buffered formalin (Appendix VII) was then gently injected into the lumen of the intestine. The whole specimen was subsequently placed in this fixative within suitably sized specimen bottles.

Tissue intended for microscopic examination was harvested from the jejunum only. A two centimetre section was collected from near the

beginning of the jejunum, 8 centimetres distal to the pyloric sphincter. A further specimen was obtained from the estimated midpoint of the jejunum and a third from the distal portion. This tissue was processed and subsequently blocked in wax using routine histological procedures (Appendix VII). Sections were cut with a rotary microtome set at 5 microns. For histological assessment 3 representative sections per section of gut were selected at intervals of 20 cuts and these were stained using haematoxylin and eosin. Some additional sections were stained using the periodic acid Schiff, diastase staining method (Appendix VIII).

MICROSCOPIC ASSESSMENT OF TISSUES

Three sections from the proximal jejunum and 3 sections from the mid jejunum were scrutinized and quantitatively assessed (Appendix IX). Specimens from the distal jejunum were examined but not assessed.

A number of indices were scored:

(1) Villus Shape

Villus shape was considered to be normal when the outline of the villus was regular and no generalized reduction in villus height was observed. It was described as abnormal when the section exhibited a generalized reduction in villus height. Major abnormality was considered to be present when villi were absent or a major reduction in villus height was present, leaving the epithelial lining a gently undulating outline.

(2) Lamina Propria

The lamina propria was subjectively assessed normal or classified as abnormal, to a minor or major degree. Minor changes consisted of dilated lymphatics or obvious oedema within up to half of the villi. Major changes consisted of these changes in most or all of the villi.

(3) Epithelium

The epithelium was described as normal if it consisted of simple columnar cells. It was assigned to a minor abnormality category where a proportion of the cells departed from this morphology, such as a change to a cuboidal shape. Major changes were deemed to be present if only flattened cells covered the villi or if the epithelium was incomplete. Ulcerations were specifically noted. Minor cellular losses at the villus tips were not regarded as abnormalities as degenerating cells are normally seen exfoliating from this site.

(4) Goblet Cells

Goblet cells were identified from their mucin content. The average number of goblet cells on the tip portion of the villi was estimated by counting the cells on one well oriented villus in each quadrant of the intestinal lumen. Cells were counted from the tip of the villus down each wall for a total distance of 0.4 mm at magnification x 400.

Three aspects of the histology of the crypts of Lieberkuhn were investigated.

(5) Crypt Morphology

These were assessed as being normal, or as having minor or major changes from normal. Thirty crypts were assessed in each section. Minor changes were a decrease in size of the crypts. This was often seen as a change to an ovoid shape rather than a tubular shape. A generalized dilation of the crypt lumen was also considered to be a minor change. Major changes were present if more than 50% of the crypts had undergone partial or complete cystic dissolution.

(6) Crypt Cell Death

This was scored as being either present or absent in each section. Assessment was based on the presence of obvious nuclear degenerative changes such as pyknosis or karyorrhexis.

(7) Mitoses in Crypts

These were noted to be absent, present, or present and multiple. This was assessed by counting mitotic figures in 30 well orientated crypts of Lieberkuhn in each section. Multiple mitoses were considered to be present if an average of greater than one mitotic figure per crypt could be identified. Only those cells obviously undergoing mitosis were included. These would be in anaphase, late metaphase or early telophase.

EXPERIMENTS

Two experiments were undertaken during the course of this study. The first of these, the toxicity study, was designed to test the

toxicity of methotrexate in rats when delivered by a 24 hour intraperitoneal infusion. This allowed the determination of the approximate LD 10 and LD 90 drug doses. Animals were admitted into these trials in groups of 5 (Table 3.1).

Dose of Methotrexate (mg/kg)	Animals
0	5
5	10
10	15
25	10
30	10
37.5	10
50	5
100	5
30 (+ Leucovorin)	10

Table 3.1: Animals entered into toxicity studies.

Control animals which received only an infusion of normal saline were also included in order to identify any adverse effects that the experimental procedure might have on the rats. An additional group of 10 rats received the LD 90 dose of methotrexate by 24 hour infusion. Immediately subsequent to this, they received a 24 hour infusion of leucovorin in an attempt to rescue them from the toxic effects of methotrexate.

Fifteen rats were used in an attempt to monitor the serum levels of methotrexate following infusion of the LD 90 dose of the drug. Blood was

collected by cardiac puncture from 3 animals at the end of the infusion period and at 4 later time intervals. (12,24,48,96 hours).

The blood was centrifuged and the serum analyzed by enzyme immunoassay. (Emit^{R}, Syva, U.S.A.). (Gushaw and Miller 1978). (Appendix X).

The second experiment investigated the effects of the predetermined doses of methotrexate on the small intestine of the rat. This experiment proceeded in two groups according to the dose of methotrexate administered.

Group 1.

These animals received the approximate LD 10 dose of methotrexate, 10 mg/kg, by intraperitoneal infusion over 24 hours. Twenty-three animals were entered into this group. These included one control animal. This was an age, sex and weight matched rat which was not subjected to the experimental conditions. A second control animal was handled identically to the others, but received a normal saline infusion rather than methotrexate. This animal was sacrificed 24 hours after the end of the infusion period. Twenty-one animals were subjected to the methotrexate infusion. These were sacrificed at intervals after the infusion period as follows:

0 hours	5 animals
12 hours	3 animals
24 hours	5 animals
48 hours	3 animals
96 hours	5 animals

Group 2.

Animals entered into this group received the estimated LD 90 dose of methotrexate, 30 mg/kg, over 24 hours by intraperitoneal infusion. Over the ensuing 24 hours the rats either received normal saline (Group 2A) or 30 mg/kg of leucovorin (Group 2B). Rats were sacrificed at intervals after the infusion period. Twenty-one animals were entered into this part of the study as follows:

	Methotrexate (2A)	Methotrexate + Leucovorin (2B)
0 hours	2	3
24 hours	2	3
96 hours	2	3
1 week	2	3

Additionally, one control animal which received only normal saline by infusion was sacrificed immediately after the end of the infusion period.

PHOTOGRAPHY

All photographic records were taken using Ilford Pan F black and white negative film (50 ASA, Ilford Ltd. England) and printed on Ilford glossy multigrade paper. Photomicrographs were taken using an Olympus photomicroscope equipment (PM 10ADS, BH2), (Olympus Optical Co. Ltd. Japan).

CHAPTER 4

RESULTS

Toxicity Studies:

In these experiments methotrexate was delivered by a 24 hour intraperitoneal infusion. The aim of this phase of the study was to determine the approximate levels of both the LD10 and LD90 of methotrexate when delivered by this technique.

Following the infusion period animals were observed daily. If an animal displayed signs of acute toxicity its condition was monitored each 12 hours. If an animal was seen to be severely ill or moribund it was immediately sacrificed and toxicity was presumed to be lethal. Rats were infused with methotrexate in groups of 5. Additional animals were entered into control groups. These were handled identically but were infused with normal saline only.

Control Group:

Five rats were entered into a control group. These animals were anaesthetized, had an indwelling catheter inserted and were placed into the experimental apparatus. They were infused with normal saline only, of the same volume and for the same duration as the experimental group. None of these animals died. On release from the apparatus they resumed normal behaviour and showed no visible signs of toxicity. A small weight loss occurred during the period in which the infusion was taking place but weight had begun to recover by the following day and each of the animals continued to gain weight during the succeeding days. Each had

surpassed his initial experimental weight after the passage of several days and subsequent growth appeared to be in keeping with the normal pattern of body weight increase expected in animals of this age. (Fig. 4.1). (Appendix XI).

100 mg/kg Methotrexate:

Since Rall (1963) felt that a dose 100 mg/kg represented the LD 50 in rats when delivered as a single dose, it was chosen as the initial test dose. None of the animals survived for more than 4 days. The rats lost an average of 4.7% of their pre-experimental body weight while in the experimental apparatus. The body weight of the animals failed to recover and fell continuously till their demise. On removal from the apparatus, the animals displayed normal behaviour including eating and drinking and grooming. After a further 24 hours the rats had ceased to eat and were less energetic. They assumed a characteristic hunched position in the cage. (Fig. 4.2). Diarrhoea was present in all rats at 48 hours after the beginning of infusion. At this time, small areas of pigmentation appeared on the feet and about the nose and eyes and the coat had lost its characteristic sheen. During the third day the animals appeared lethargic and weak. Drinking ceased and the animals appeared malnourished. Sickly animals did not show any signs of recovery and were sacrificed or died. (Fig. 4.3, 4.4).

50 mg/kg Methotrexate:

Acting on the findings of the 100 mg/kg group, the dose of methotrexate was halved to 50 mg/kg infused over 24 hours. Again no

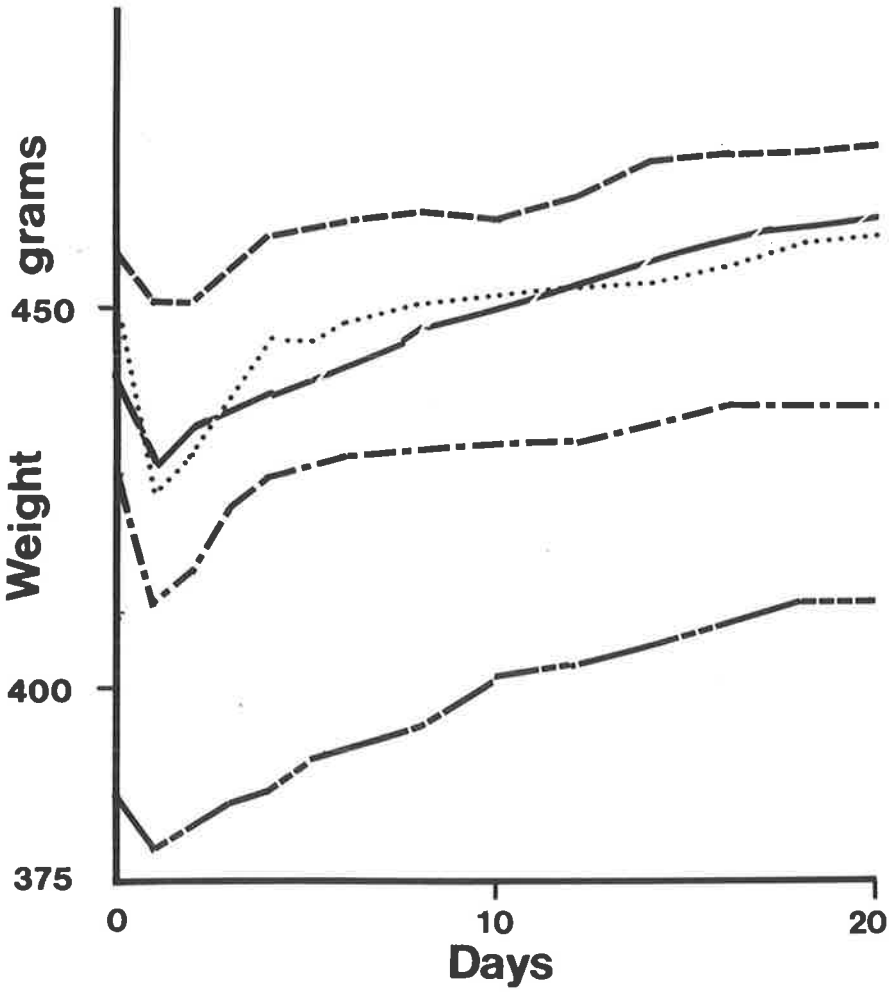


Figure 4.1: Weight changes in animals administered normal saline by 24 hour intraperitoneal infusion.

Figure 4.2: Rat 24 hours after cessation of an infusion of 100 mg/kg of methotrexate.
Note: (1) Hunched position of animal.
(2) Dull irregular coat.

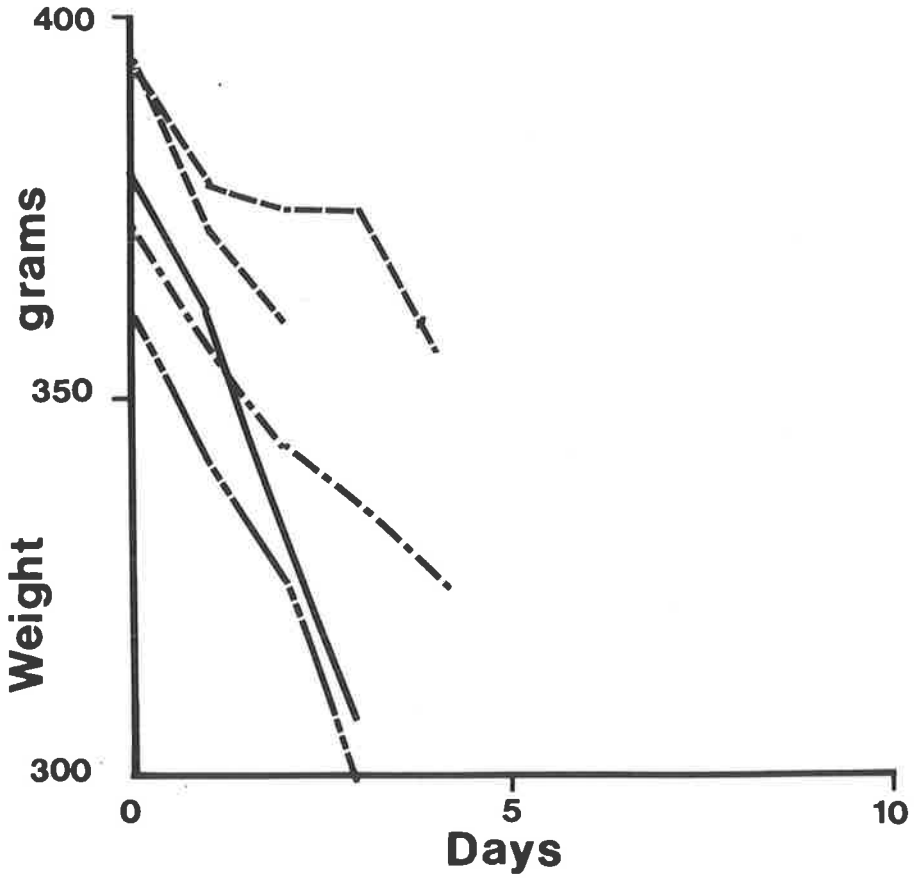


Figure 4.3: Weight changes in animals administered 100 mg/kg of methotrexate.

Figure 4.4: Rat prior to sacrifice 3 days after receiving 100 mg/kg of methotrexate.
Note: Pigmented areas on nose and around eyes.

animals survived this dose, all being dead by the beginning of the fifth day from the commencement of infusion. Two animals were sacrificed and 3 animals died suddenly. Average survival time was 3 days which was slightly less than that of the 100 mg/kg group (3.2 days). Each of these animals exhibited signs of toxicity similar in course and extent to the higher dose group. Again the impresssion was that death was due to the effects of acute diarrhoea and accompanying dehydration. Weight loss the day before death averaged 10% of pre-operative body weight. (Fig. 4.5).

25 mg/kg Methotrexate:

Ten animals were subjected to this infusion dose. The ultimate toxicity in this group was variable. Six of the 10 animals recovered from this dose. Each of the rats lost weight following the infusion. In 4 rats the weight loss was progressive and terminal. The average time of survival for these 4 rats was 7.5 days. The surviving rats suffered varying degrees of toxicity and lost weight for several days before ultimately recovering. A trough in their body weights occurred from the fourth to the eighth day. (Fig. 4.6). The least affected animal had a trough body weight loss of only 9%. All rats in this group exhibited a degree of anorexia and diarrhoea. This was obvious by day 3 to 4 but the severity and duration of these signs were variable. In those destined to survive, the period during which loose stools were apparent was only 2 to 3 days. Food intake increased after 5 to 6 days and this activity coincided with the beginnings of recovery of body weights. By the end of the observation period of 20 days, surviving animals appeared normal in all respects and all had recovered to near their pre-experimental body weights (some having exceeded them).

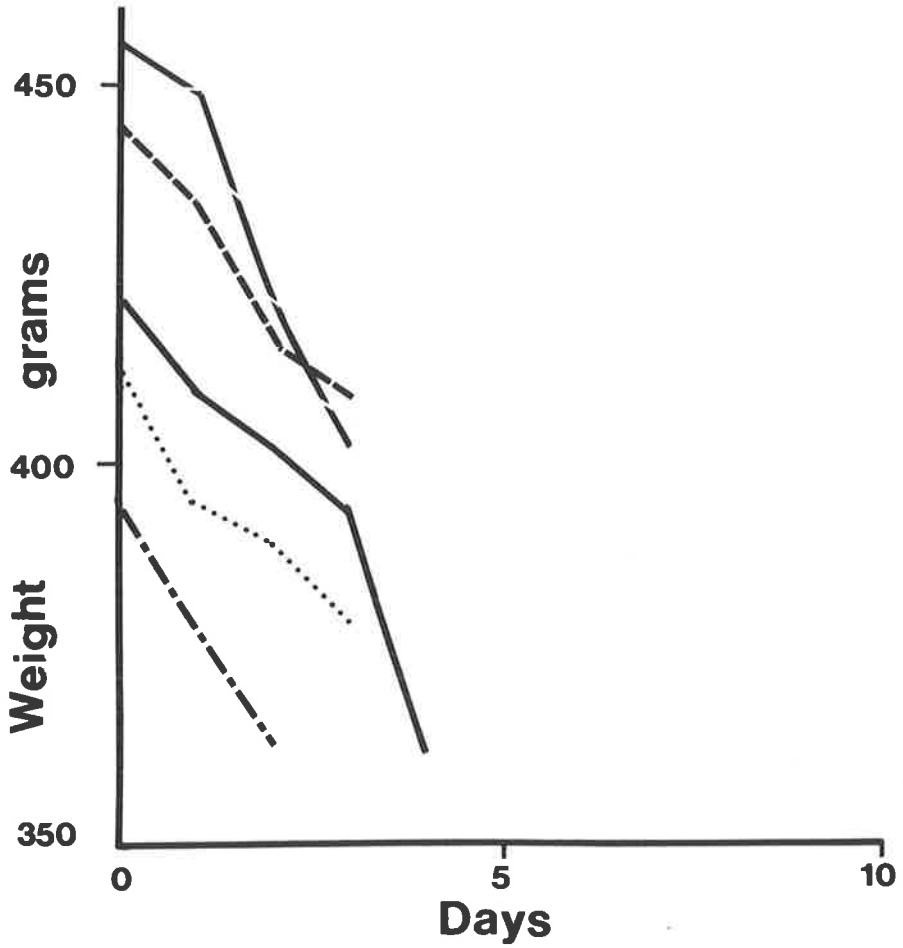


Figure 4.5: Weight changes in animals administered 50 mg/kg of methotrexate.

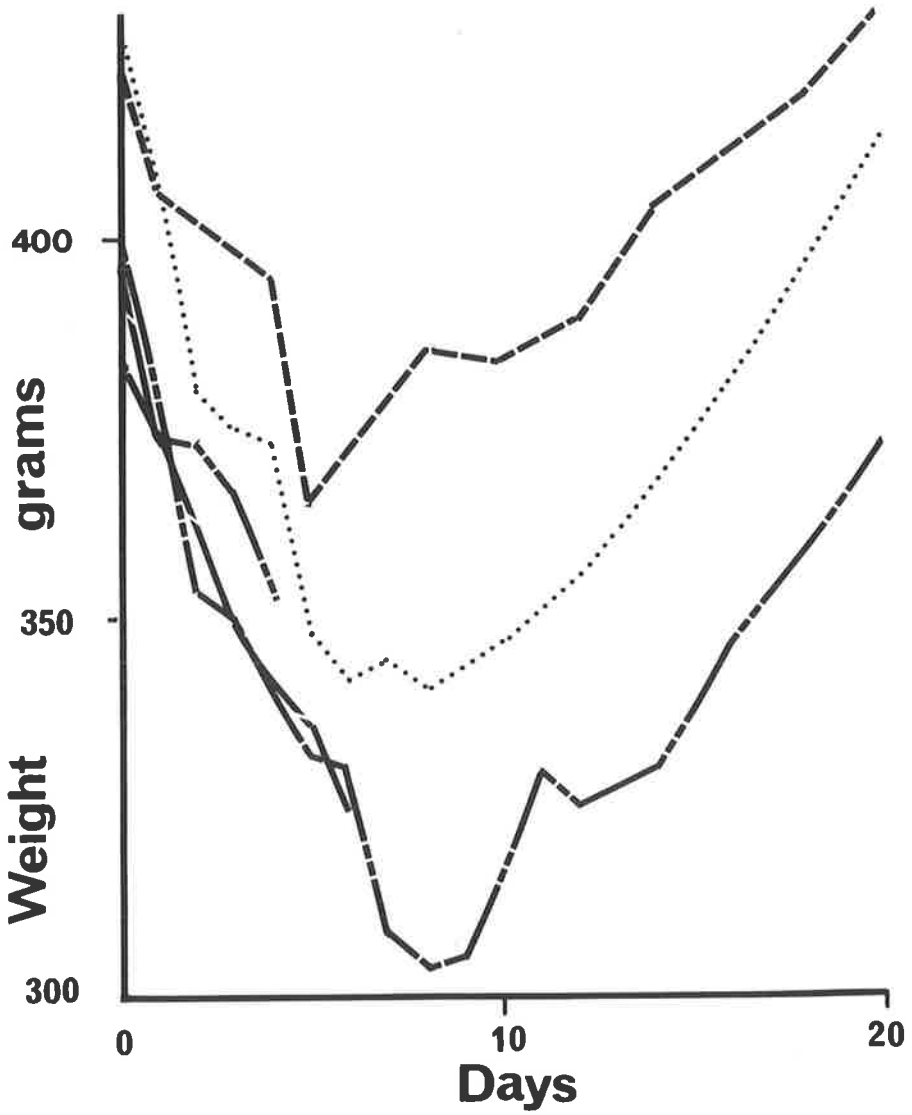


Figure 4.6: Weight changes in representative animals administered 25 mg/kg of methotrexate.

10 mg/kg Methotrexate:

Again there was variability in the response achieved. Out of 15 animals entered into this group, 2 apparently succumbed to the toxic effects of the drug (Fig. 4.7). These animals suffered a gradual decline in health noticeable from the second post-infusion day and survived for 7 and 8 days respectively. The latter animal had started to gain weight and had solid stools at the time of its death, so its demise may have been caused by toxicity other than gastrointestinal. An autopsy was not performed.

In surviving rats, the toxic effects were generally milder than those seen in the previous higher doses. All animals lost some weight in the operative and postoperative period. Weight was typically lost until the fifth to ninth days and ranged from 7.9% to 18.5% of original body weight. The surviving rats developed mild diarrhoea which was transitory, usually lasting till only the third or fourth day. Anorexia was profound in most animals during the second and third days after which food intake gradually increased to normal levels. The general condition of these animals remained good. There were only a few cutaneous pigmented lesions visible and the strength and activity levels of the animals were only briefly reduced.

5 mg/kg Methotrexate:

Ten animals received this dose with there being no resultant mortality. The morbidity was variable. Several of the animals suffered very little weight loss and developed no diarrhoea or other obvious features of toxicity (Fig. 4.8). Others lost weight until up to the

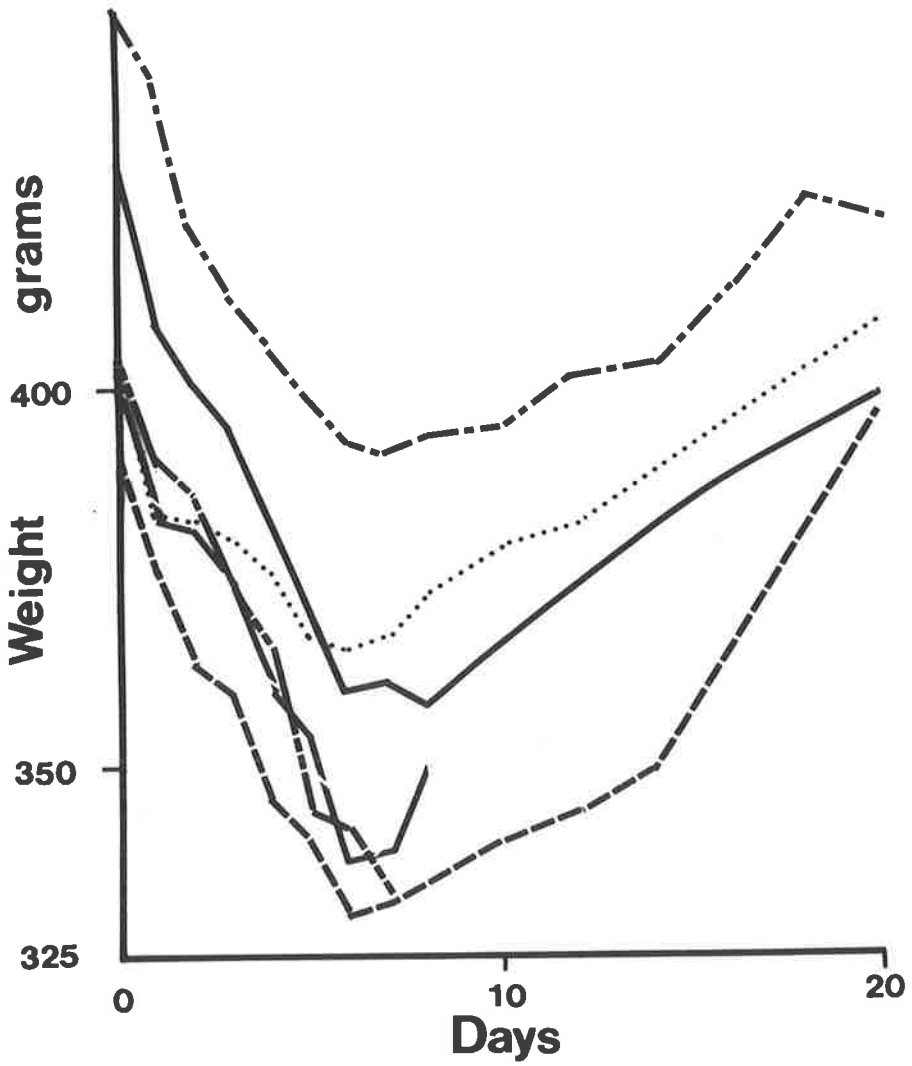


Figure 4.7: Weight changes of representative animals administered 10 mg/kg of methotrexate.

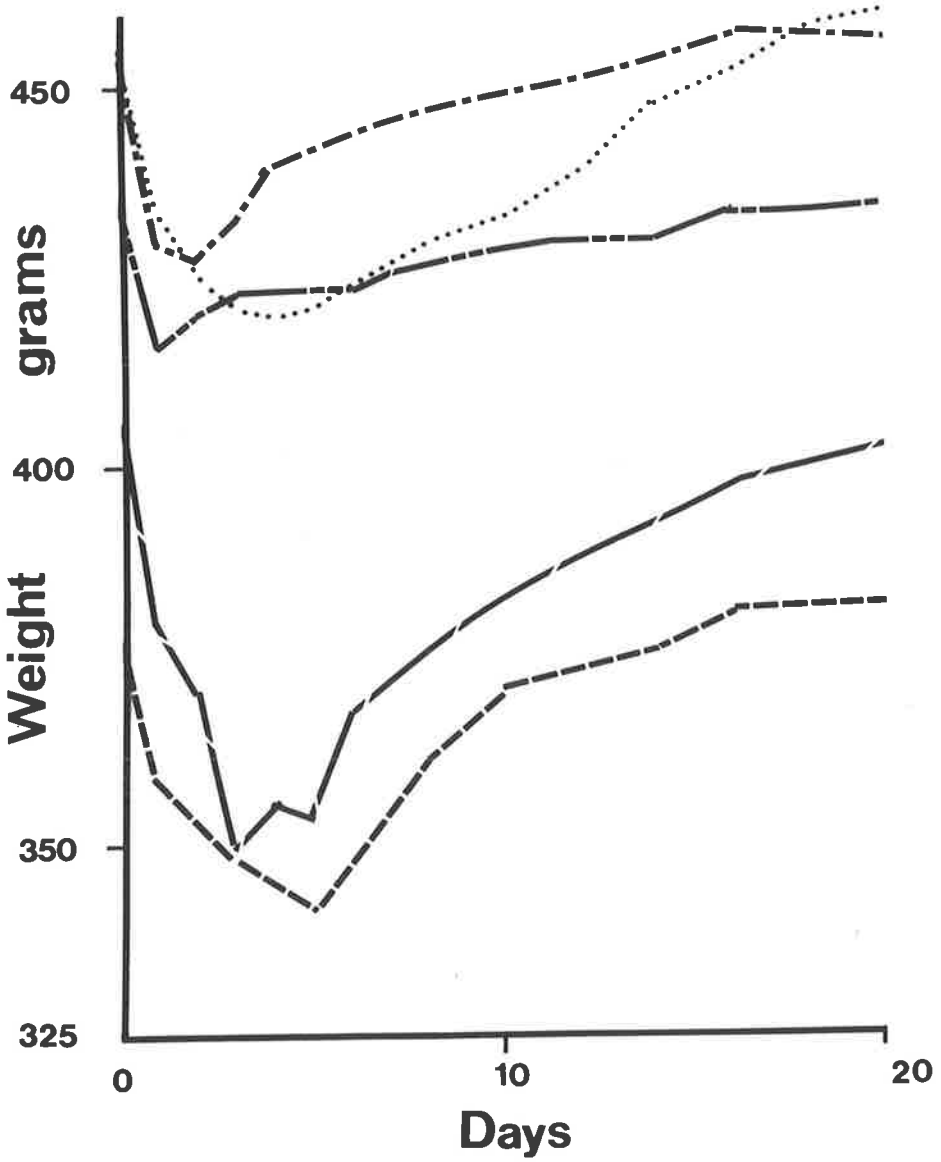


Figure 4.8: Weight changes in representative animals administered 5 mg/kg of methotrexate.

fifth day, but in all cases weight loss was mild, ranging from 3 to 13.5% (average 6.8%). Several animals developed mild transitory diarrhoea but none of these became debilitated. The rats appeared to continue eating throughout the observation period.

37.5 mg/kg Methotrexate:

37.5 mg/kg was administered to a group of 10 rats. This dose proved to be uniformly fatal. The course of intoxication was similar to that seen following the 50 mg/kg dose. No animal survived for more than 9 days. Average survival time was 4.5 days, a little longer than the 50 mg/kg group. (Fig. 4.9).

30 mg/kg Methotrexate:

A further 10 rats were subjected to a dose of 30 mg/kg of methotrexate. Of these animals 9 perished and 1 survived. The average time of survival of those rats which died was 5 days. All of these rats progressively lost weight from the time of experimentation. Diarrhoea was present on the second or third post infusion day. In some cases the rats had recovered from this before their deaths. The surviving animal lost a maximum of 10% of its body weight before beginning to recover on the eighth day. (Fig. 4.10).

Summary:

The results of the toxicity studies are summarized in Table 4.1 and represented graphically in Fig. 4.11.

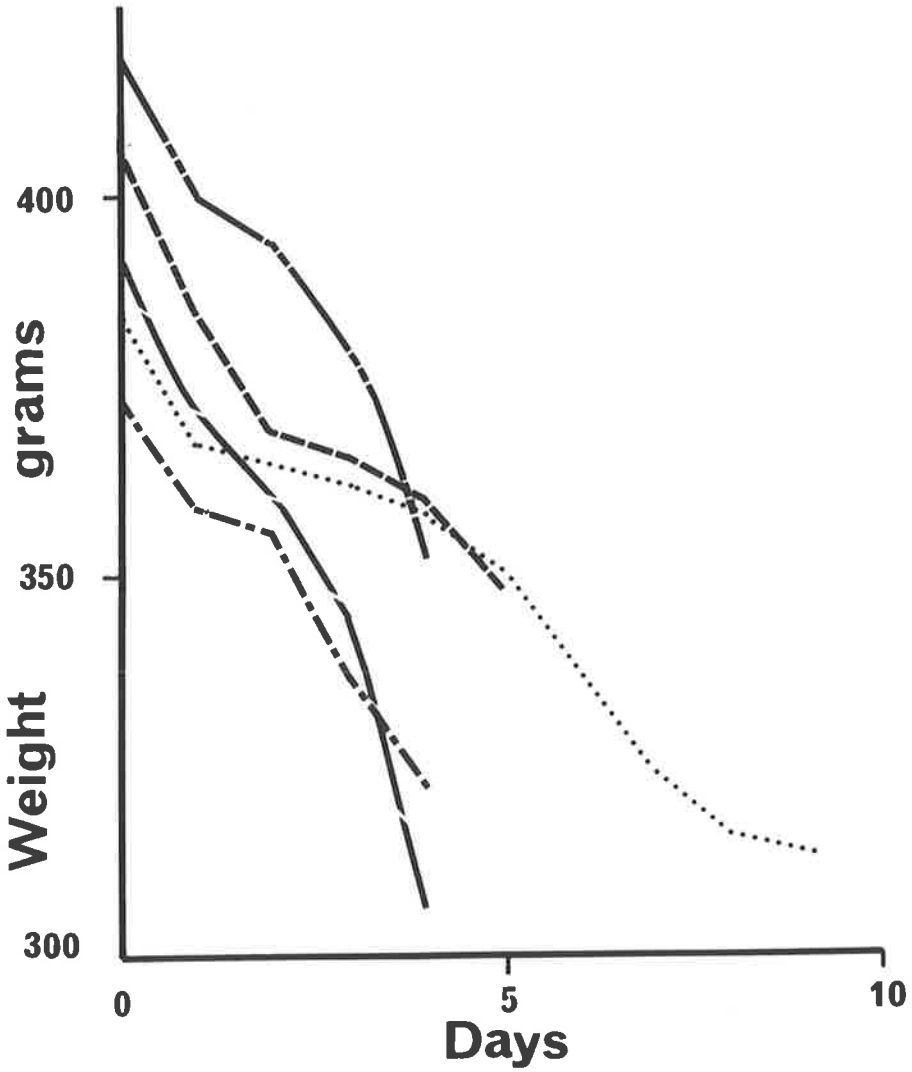


Figure 4.9: Weight changes in representative animals administered 37.5 mg/kg of methotrexate

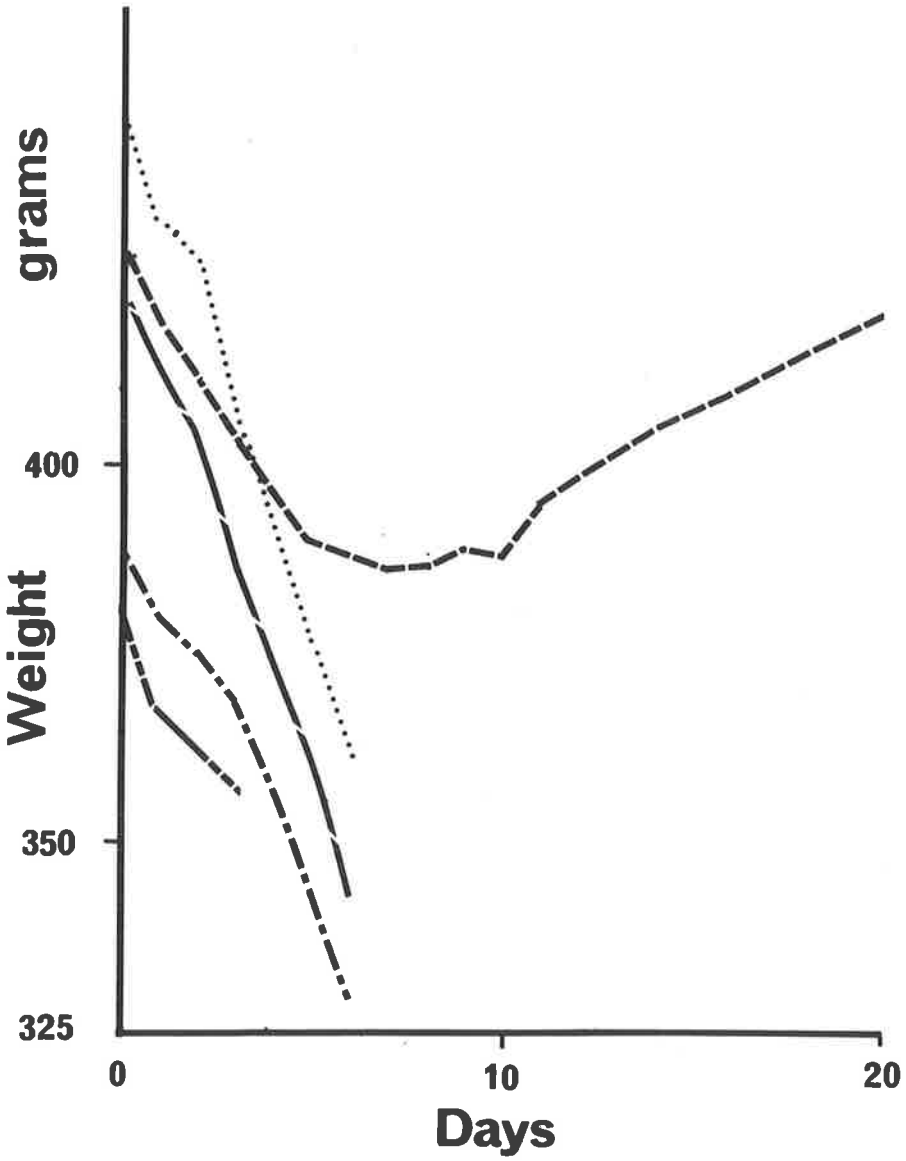


Figure 4.10: Weight changes in representative animals administered 30 mg/kg of methotrexate.

Dose	Animals	Deceased	%
0	5	0	0
5	10	0	0
10	15	2	13.5
25	10	4	40
30	10	9	90
37.5	10	10	100
50	5	5	100
100	5	5	100

Table 4.1: Fate of animals infused with various doses of methotrexate.

From these results it was concluded that 10 mg/kg could be considered an approximation for the LD 10 of methotrexate, delivered by this method in these animals. Similarly, an infusion dose of 30 mg/kg was considered to be a reasonable estimation for the LD 90.

Serum Methotrexate Assay:

Serum methotrexate levels were measured following the infusion of 30 mg/kg of the drug. Blood was collected by cardiac puncture from 3 animals at the end of the infusion period and at 4 later time intervals (Table 4.2). Cardiac puncture was unsuccessful in one animal at 48 hours and a further animal failed to survive for 96 hours. At the end of the infusion period the maximum level of methotrexate recorded was 7.4×10^{-6}M. By 48 hours post infusion methotrexate was no longer detectable in the serum.

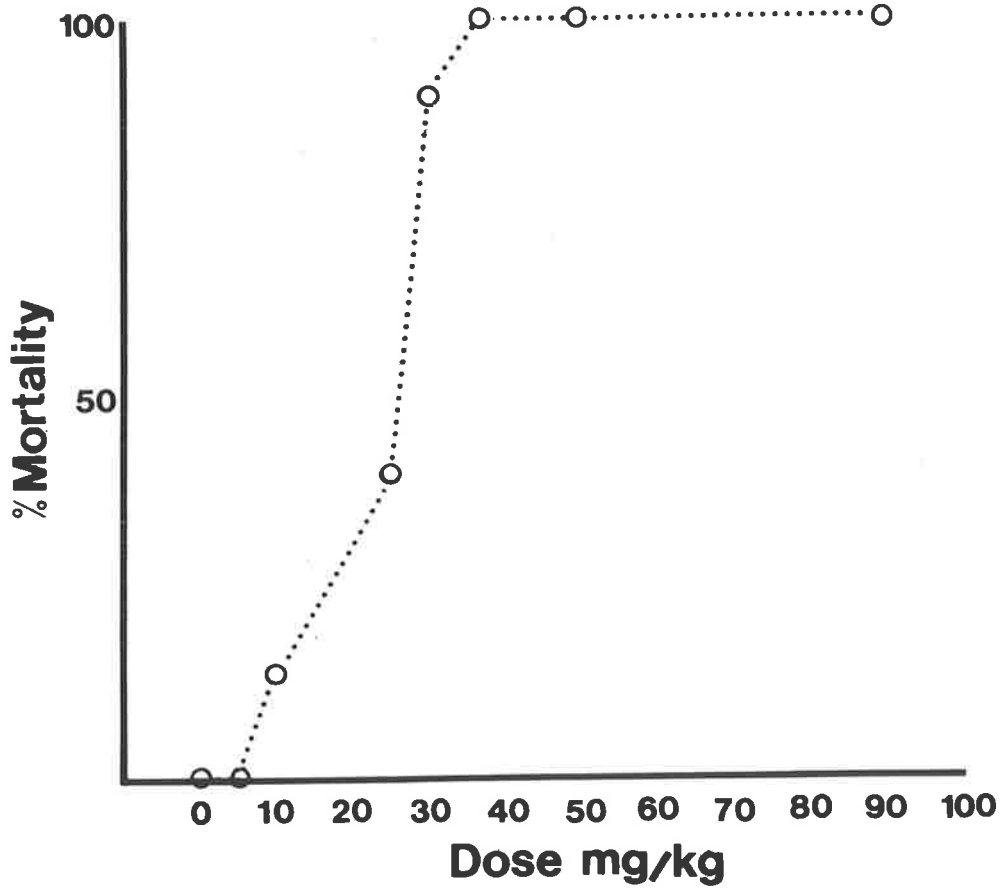


Figure 4.11: Percentage mortality of rats following the infusion of various doses of methotrexate.

Hours (post infusion)	Sample	M/1 x 10^{-6}
96	A1	0
	A2	0
48	B1	0
	B2	0
24	C1	0.14
	C2	0.05
	C3	0.3
12	D1	1.20
	D2	0.80
	D3	1.10
0	E1	0.71
	E2	7.40
	E3	2.6

Table 4.2: Serum methotrexate levels following infusion of 30 mg/kg over 24 hours.

Trial Leucovorin Rescue:

Ten animals received a 24 hour infusion of 30 mg/kg of methotrexate, followed by 30 mg/kg of leucovorin over the succeeding 24 hours. Eight animals survived this regime. The other rats succumbed on days 4 and 8 respectively. The surviving animals exhibited variable signs of toxicity. At the time of maximum weight loss animals had lost an averge of 14.5% of their body weight (range 9.8-19.5%) and this was seen from the fourth to the seventh day after the commencement of the infusion (Fig. 4.12). Each of the animals developed diarrhoea which persisted for several days and which varied in severity from very mild to quite severe. The more seriously affected animals also became

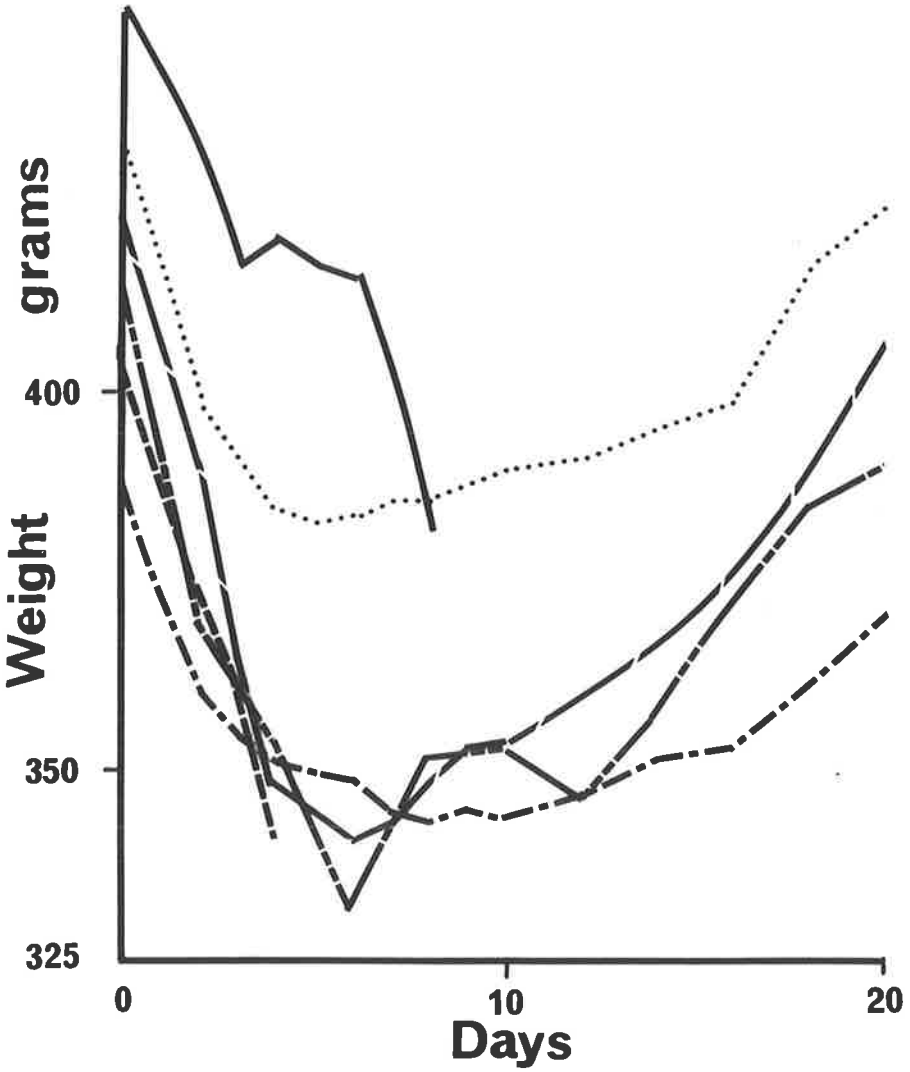


Figure 4.12: Weight changes in representative animals administered 30 mg/kg of methotrexate followed by 30 mg/kg of leucovorin.

anorexic during this time, but others continued to eat. All surviving animals continued to drink.

SMALL INTESTINAL STUDIES

Group 1:

There was no mortality amongst this group of animals. Each survived until its scheduled time of sacrifice. The behaviour and toxic effects observed on gross observation of these animals were consistent with those seen in the animals which received 10 mg/kg of methotrexate during the toxicity trials.

The tissue removed from these animals was unremarkable on gross examination. From 24 hours following the infusion period the intestinal tract contained loose stools, but by 96 hours the rectum again contained well formed solid stools.

UNTREATED CONTROL ANIMAL

Haematoxylin and eosin stained sections of the jejunum of this animal displayed a histological picture characteristic of normal tissues of this area. (Fig. 4.13).

The muscular layers encircled the submucosa and mucosa in a continuous eosinophilic layer of variable thickness. Blood and lymphatic vessels were seen to penetrate the submucosa. At the bases of the villi, crypts of Lieberkuhn were conspicuously massed. A number of these were obviously in communication with the villus walls and intestinal lumen,

whilst others looked to be blind owing to the plane of section (Fig. 4.14). Most of the crypts had no lumen space but some had a narrow visible lumen containing mucous. The crypts ranged in shape from ovoid, to tubular in the case of those visibly continuous with the villus wall. These had a depth which was several times their diameter. Some crypts contained large numbers of goblet cells (Fig. 4.14), their cytoplasm grossly dilated with mucin. Other crypts displayed few of these cells. Most crypts contained cells which were undergoing mitosis (Fig. 4.15). Mitotic figures were not seen out of the crypts proper on the walls of the villi. In the depths of the crypts the characteristic eosinophilic granules of paneth cells were seen.

The villi were coated by an orderly array of epithelial cells comprising a single layer of absorbtive and goblet cells (Fig. 4.16). A number of goblet cells could be seen discharging their mucous contents into the intestinal lumen. The absorbtive cells were of columnar shape with apically situated ovoid nuclei. The epithelial cells formed an unbroken covering to the villus. The villi were of variable shape and length owing to differences in the plane of section (Fig. 4.13). The tips of the villi were generally intact but occasional areas of cellular exfoliation could be identified (Fig. 4.17).

The lamina propria contained a number of cells with prominant basophilic nuclei. These were enmeshed in a fine fibrous tissue stroma. Lymphocytes appeared to be the most numerous cells in the lamina propria. Polymorphs, macrophages and plasma cells were also identified. A few lymphocytes were situated within the epithelium (Fig. 4.16). Lacteals were visible within some villi.

There was a significant variation in the architecture of the epithelial lining depending on the site of tissue sampling. Both villus height and crypt size were greater on average in the more proximal part of the jejunum (adjacent to the duodenum) than in the terminal portion (adjacent to the ileum). Villi appeared to be less numerous more distally. The diameter of the gut also increased from the duodenum to the ileum.

CONTROL, SALINE INFUSION

On examination of the jejunal tissues, no obvious histological differences could be identified between this and the control animal.

Villi again were intact and of optimal length and form (Fig. 4.18). The epithelial cells were unchanged in configuration and the villi tips showed no abnormal cellular loss (Fig. 4.19).

Group 1. Zero hours:

The general architecture of the jejunum was intact in all of the specimens examined from this group (Fig. 4.20). Some minor structural changes were seen and these varied in degree between the animals examined. In general, villus size and shape was not obviously changed from that seen in control animal tissue. (Fig. 4.22). Likewise, the epithelial cells maintained their columnar shape and their staining characteristics. Goblet cells were evident. The lamina propria was undisturbed in one animal (Fig. 4.22), but the others showed variations from normal. In some areas there was some dilatation of lacteals (Fig. 4.23) but the most abberent sections demonstrated a considerable

oedema of many villus tips (Fig. 4.21), the terminal epithelium appearing to split from the basement membrane. Lymphatic channels at the bases of the crypts were also conspicuously dilated in the same sections (Fig. 4.24).

The crypts of Lieberkuhn were the site of the most significant changes. The severity of changes was variable, but the number of cells in the crypts and the average size of the crypts appeared to be reduced in all sections (Fig. 4.25). Many crypts were shaped round or ovoid in shape rather than tubular and few could be traced to the gut lumen. Mitoses were not in evidence in these sections. Furthermore, dead or dying cells were seen in nearly all of the crypts examined (Fig. 4.26). The most obvious evidence for this came from examination of the nucleus. Some nuclei were pyknotic, others demonstrated fragmentation of the chromatin and loss of the nuclear membrane and could be considered to be undergoing karyorrhexis. Cytoplasmic vacuolation was also occurring in some cells, although this was less obvious owing to the proximity of goblet cells. Paneth cells appeared normal. The muscular layers were intact and showed no obvious changes.

Group 1. Twelve Hours:

Specimens from animals sacrificed 12 hours after the end of the infusion period displayed similar changes to those seen in the previous group. Villi still attained a good height in all sections examined. The epithelial cells remained neatly arranged on the walls of the villi and these conformed with the usual simple columnar shape. Mucous cells were numerous and appeared to be actively excreting. Epithelial cell loss was more conspicuous in these sections, resulting in small defects toward

the tips of the villi (Fig. 4.28). Examination of the lamina propria failed to reveal any abnormalities in cellular composition. Inflammatory cell numbers were not obviously increased or changed in distribution. Oedema was present at the tips of a proportion of the villi and marked lymphatic dilatation was a feature of the bases of the villi in all but one animal.

The most obvious histological changes were observed in the crypts of Lieberkuhn. The area occupied by the crypts was decreased. Most were of ovoid or circular shape rather than tubular (Fig. 4.28). The status of the crypt cells varied. A considerable proportion of them were degenerating, being in various stages of nuclear disintegration and cytoplasmic vacuolation (Fig. 4.29). In some of the crypts the lumen appeared dilated and empty. In others, degeneration and liquefaction of a proportion of the cells left a void in the wall of the crypt. No mitotic figures were identified in any of the crypts of this group of animals.

One animal showed less severe changes on histological examination (Fig. 4.27). Notably, the lumen of the jejunum of this animal was compacted with food particles. These had compresssed the tips of the otherwise tall villi. Preparation of the tissues had failed to dislodge the food in this case. Little oedema was seen in these sections and the structure of crypts had changed less, although degenerating cells were visible in most crypts.

Group 1. Twenty-four Hours:

As in previous groups, the most dramatic changes seen in specimens from this time were observed in the lamina propria and in the crypts of Lieberkuhn. The types of changes noted were similar in all sections examined, although there was some variability in the severity of the changes between animals.

In all examples the epithelium retained its integrity, although prominant clefts were seen appearing to undermine portions of lamina propria (Fig. 4.32). The epithelial cells had retained their palisaded arrangement on some of the villi, whilst on others where the epithelial outline was more irregular, the absorbtive cells also appeared irregular in shape. Some terminal epithelial cells displayed vacuolation of the cytoplasm (Fig. 4.31). Goblet cells were present in some crypts and on the walls of each villus. On some villi, dilated mucous cells were the predominant cell type present (Fig. 4.32). The gross form of the villi was still evident (Fig. 4.30). However, the villi had a more irregular outline and less symmetry of shape than that observed in control animals. A subjective impression was that the height of the villi had decreased throughout the sections. On a few villi a large group of cells appeared to be splitting from the villus tip (Fig. 4.32). Additionally, loss of individual cells was a feature of many villus tips.

Oedema of the lamina propria was present in all specimens from this group. The oedema was variable in extent throughout the sections. In some areas it was marked at the villus tips. In severely affected areas a major proportion of the lamina propria comprised oedematous spaces. Lymph vessels in the lamina propria were often grossly dilated and seen

to be in continuity with the lacteal extending to the tip of the villus (Fig. 4.30). An apparently increased lymphocyte population was seen in some sections (Fig. 4.31).

The crypts of Lieberkuhn exhibited the presence of dying and degenerating cells and the area occupied by the crypts was reduced, in comparison to that seen in those animals subjected to earlier sacrifice. Crypts were of several morphological types. There remained some in which the structure was not noticeably aberrant, although these were reduced in proportion. A number of crypts had dilated lumina surrounded by cells ranging from apparently normal, to dying cells and disintegrating cells (Fig. 4.33). Other deranged crypts consisted of only a central lumen surrounded by flattened and degenerating cells. The lumen was either empty or contained cellular debris. The few crypts which were still in communication with the gut lumen barely invaginated the area between adjacent villi. No mitotic activity was in evidence in these sections. Paneth cells appeared unchanged in structure or situation until the terminal stages of crypt dissolution.

Group 1. Forty-eight Hours:

At this time villus form, epithelial cell morphology and crypt morphology were all markedly disrupted. The villi no longer retained their fingerlike shape but rather were flattened and consequently decreased in height (Fig. 4.34).

The epithelium was grossly intact in all of the sections examined. However, in some areas it could be seen lifting from the lamina propria. In areas featuring irregularly shaped villi, single cells and larger clumps of cells were sloughing off the villus tips leaving micro-ulcerations, but where the villi had become grossly flattened, the epithelial covering appeared to be more regular and cell loss was minimal (Fig. 4.36).

Epithelial cells exhibited a change in shape. In some areas the epithelial cells were of an irregular cuboidal shape (Fig. 4.36). In other areas only a single layer of flattened cells constituted the epithelium. Goblet cell numbers were markedly reduced and were absent on some villi.

The striking feature of the lamina propria was its oedematous nature and the prominant lymphatic structures (Fig. 4.35). Dilated lymphatic channels were conspicuous in all areas and at their most dramatic, contributed the major proportion to the villus area, extending from the intestinal glands to near the tips of the villus (Fig. 4.35). It appeared that the dissolution of some crypts had contributed to these spaces as they had coalesced with the lymphatic channels. The only change to the cellular makeup of the lamina propria was a large increase in the number of extravasated erythrocytes, indicative of haemorrhage. A large peyers patch present in one section retained its basic structure and its population of lymphocytes did not appear to be abnormal.

The crypts of Lieberkuhn were less numerous than in sections from animals sacrificed at earlier times and, as was noted in the previous groups, their structure varied. Few crypts still had a tubular shape in

cross-section and very few were in communication with the lumen. At the base of the villus wall the cells were generally indistinguishable from the rest of the epithelial covering (Fig. 4.37). Most remaining crypts were of circular or ovoid shape with cells clustered around a central lumen. The crypt cells had variable morphology. Some were dead or in the process of degenerating. Others were viable, their appearance being consistent with that of normal cells. A small number of mitotically active cells were identified in the crypts of one of the animals in this group. Other crypts had completely degenerated into microcystic like structures surrounded by a few flattened cells with deeply stained flattened nuclei (Fig. 4.37). The lumina of these structures contained cellular debris or were empty.

Group 1. Ninety-six Hours:

The mucosa of specimens from this group was considerably changed from that seen in 48 hour specimens. A number of villi had returned to what was considered optimal dimensions (Fig. 4.38). Interspersed between these villi were a number of shorter ones. The epithelium showed some variations in structure. Most areas were covered by well orientated simple columnar epithelium (Fig. 4.39), whilst other areas showed cuboidal shaped cells (Fig. 4.40). Cells on the tips of villi were more irregular. Well formed goblet cells were interspersed between absorbtive cells.

The lamina propria exhibited very mild oedema at the villus tips (Fig. 4.40). A number of villi contained dilated lacteals. More peripherally the lymphatic vessels were only rarely dilated. Some additional vacuolated areas in the lamina propria were noted. These were

interpreted as being the vacuolated remnants of crypts of Lieberkuhn. (Fig. 4.40). Erythrocytes were numerous in the lamina propria, randomly scattered between connective tissue cells.

Most of the intestinal glands had recovered their tubular shape and featured groups of paneth cells at their bases. (Fig. 4.41). Many crypts still had a dilated lumen but the lining cells were of healthy appearance, with regular ovoid nuclei and pale staining granular cytoplasm. Many of the crypt cells were in various active stages of the mitotic cycle (Fig. 4.39). These mitotic figures were most common in the depths of the crypts, but could also be seen as the crypts opened onto the villus wall. Some degenerate crypts were noted. (Fig. 4.41). These were surrounded by a single layer of flattened atypical cells and contained cellular debris and occasionally lymphocytes.

Groups 2A and 2B:

Tissue was not available for examination from several of these animals owing to early death from drug toxicity. No unrescued animals survived into the 96 hour time period. One animal from this group was sacrificed early, at 48 hours, as it was moribund. One animal which had received the rescue agent failed to survive the prescribed 7 days before examination. Other animals were sacrificed as scheduled.

Some gross features of the gastrointestinal specimens were worthy of note. The gut from each of the unrescued animals contained no solid faeces. These specimens had foul smelling watery contents. The diameter of the jejunum and ileum appeared to be greater than normal in comparison with the duodenal diameter. The length of the intestines

appeared to be shortened. The gut from the rescued group also showed these changes at 0 and 24 hours. Rescued animals had produced solid faeces by 96 hours and one week post infusion.

CONTROL ANIMAL, SALINE INFUSION

This animal was subject to experimental conditions and infused with normal saline only. It was sacrificed 48 hours after the commencement of the infusion. Jejunal tissue from this animal displayed anatomy consistant in appearance with that of the previous control animals. Villi were of optimal height and form and the epithelial covering was intact. Epithelial cells displayed their classical shape and arrangement.

The lamina propria exhibited no oedema and the lacteals were hard to identify. There was no undue inflammatory cell component to the cell population. The crypts of Lieberkuhn were well formed and deep, with no evidence of cell death being present. Mitotic figures were obvious in a number of crypts.

Group 2a. Zero Hours:

These animals were sacrificed 24 hours after the cessation of their methotrexate infusion. They received normal saline for the intervening 24 hours. Microscopic examination of sections revealed marked degenerative changes. As in the previous series of animals the changes remained confined to those layers of the jejunum medial to the muscle layers. The form of the villi was basically intact although the tips of many of them were flattened and irregular, so that a generalized

reduction in villus height had occurred. (Fig. 4.42). The continuity of the epithelium was intact throughout these sections, but the epithelial layer was quite irregular in outline. The epithelial cells exhibited pronounced morphological change, evidenced by transformation from columnar to cuboidal shape (Fig. 4.43). The cuboidal cells were characterized by ovoid nuclei situated in the centre of each cell rather than near the apex. Goblet cells were still present. Some of these were interspersed between the cuboidal cells near the villus tips, but there was a greater concentration of goblet cells further down the villus wall and particularly at the bases of the villi. On some villus tips there was evidence of epithelial cell exfoliation. In some areas larger segments of epithelium appeared to be sloughing. In these regions the epithelium had split from the basement membrane, leaving a space between the epithelium and the underlying lamina propria (Fig. 4.43). Blood vessels within the lamina propria appeared to be dilated. A number of lacteals were also dilated and these were in continuity with prominant lymphatic vessels more peripherally.

The number of intestinal glands was reduced compared with normal. Those glands remaining showed diminution in size and depth. Remaining crypts exhibited widely dilated lumina containing fibrillar material and cell debris (Fig. 4.44). In some areas, the sites previously occupied by crypts at the base of the villus wall had been replaced by a single layer of epithelial cells (Fig. 4.43). The cells comprising the crypts were in various stages of degeneration. In some, pyknotic nuclei were identified. Other nuclei exhibited fragmentation of the chromatin. Only a few crypts contained a population of normal cells. Other crypts were surrounded by a small number of flattened cells (Fig. 4.44). No mitoses were seen in specimens from this time period.

Group 2A. Twenty-four Hours:

Major changes from normal were seen in the mucosal tissues of these animals. Villus height was reduced in all sections examined and the villi were flattened rather than fingerlike in shape (Fig. 4.45). No villi exhibiting normal morphology remained.

The mucosal epithelium was thin (Fig. 4.47). In some areas the epithelium appeared to be fragmenting (Fig. 4.46) and micro-ulcerations were present, but in no section was a large area of ulceration apparent. The number of epithelial cells was considerably reduced from normal and those remaining had markedly altered morphology. These were squamous-like cells with flattened nuclei but the detail of their morphology was variable (Fig. 4.48). A population of irregular cuboidal cells was also present.

The lamina propria displayed striking changes from normal. Extravasation of erythrocytes consistant with haemorrhage was noted. The inflammatory cell population was altered. These were very prominant towards the villus tips and subjectively appeared to be increased in numbers (Fig. 4.45). The peripheral portion of the lamina propria contained grossly dilated lymphatic vessels which comprised a large proportion of this tissue (Fig. 4.46).

The crypts of Lieberkuhn had dilated lumen surrounded by cells of variable morphology. Many of these were flattened and contained elongated nuclei. Few crypts had a population of normal appearing cells. No goblet cells were present in the crypts and very few were on the villi (Fig. 4.47). The cellular lining of the crypts appeared in some

areas to be breaking down, so that a microcystic crypt communicated with a lymphatic vessel (Fig. 4.47). The size and number of the intestinal glands was reduced.

Group 2A. Forty-eight Hours:

Due to the death of other animals in this group, only one animal was available for histological assessment. Microscopic examination of this gut tissue demonstrated the most severe atrophy of the jejunal mucosa seen in this series (Fig. 4.49). No villi could be identified. The gut lumen was surrounded by an undulating lining of severely atrophic epithelium which was incomplete in places, leaving areas of ulceration (Fig. 4.50). The epithelial cells were flattened but otherwise variable in shape. The nuclei of these cells also varied in size and shape (Fig. 4.51). The lamina propria contained many free red blood cells and a prominant lymphocyte population. Crypts of Lieberkuhn were situated within the lamina propria rather than being in communication with the jejunal lumen. The majority of these had degenerated into small ovoid cyst-like structures, either empty of contents or, containing dead cells undergoing disintegration (Fig. 4.52). A small number of the crypts contained a population of viable appearing cells. Despite the major disruption of the mucosa, the submucosa and muscular layers remained apparently unchanged from normal.

Group 2B. Zero Hours:

These animals were sacrificed for examination immediately after the leucovorin rescue period. The villi of the jejunum of these animals appeared to be of optimal height (Fig. 4.53). Some cellular loss was

evident from a number of villus tips. The epithelium was composed of columnar cells and a prominant population of goblet cells. (Fig. 4.54). Some oedema was present at the tips of villi and a number of lacteals were prominant. Lymphatic vessels extending towards the submucosa were dilated (Fig. 4.55). The crypts of Lieberkuhn were generally reduced in size and appeared to contain fewer cells than normal. The crypts were of the two normal types. Tubular ones communicated with the intestinal lumen. Ovoid or circlar ones deeper in the tissues contained few goblet cells. The general architecture of the crypts were still intact (Fig. 4.55).

The crypts of Lieberkuhn contained a few scattered mitoses in two of the animals in this group. Paneth cells remained normal. Many of the other cells within the crypts showed changes culminating in cell death and fragmentation. A number of cells showed nuclear changes. Nuclei were irregular in shape, some being enlarged compared to the cytoplasmic area. Others had become reduced in size and were very darkly stained and pyknotic. The cytoplasm also showed changes. Some were swollen and vacuolated and inclusion bodies could be seen in others.

Group 2B. Twenty-four Hours:

All sections examined in this group showed considerable structural and cellular changes (Fig. 4.50). There was a marked decrease in the villus height, villus area and area occupied by the intestinal glands. These features were of variable severity.

The tips of the villi were flattened and the villi were irregular in outline. The lamina propria contained a prominant population of

lymphocytes and red blood cells. Most of the erythrocytes were seen to be confined to blood vessels. The area occupied by the lamina propria did not appear to be reduced but a large proportion of it was taken up by large lymphatic "lakes" (Fig. 4.56). Oedematous spaces were also obvious on the villus tips under the epithelium.

The epithelium was irregular in shape and outline. The relative continuity of the epithelium was confirmed by identifying the continuous line of goblet cells visible in PAS stained sections (Fig. 4.57). The goblet cells were massed in the lowest part of the villus wall but were virtually absent from the crypts. No columnar cells remained, these being replaced by cuboidal cells and others of a more irregular shape (Fig. 4.58).

The intestinal glands were considerably reduced in size. Few of the crypts were in communication with the lumen of the gut. Most crypts were ovoid in shape and had a dilated lumen which was either empty or contained dead cell components or occasional lymphocytes. There was great diversity in the status of the crypt cells. Some crypts were surrounded only by flattened cells with flattened nuclei. Other crypts contained a larger cell population composed of a mixture of normal cells, dead cells and some degenerating cells. Intermingled with these cells were a small number of cells undergoing mitosis (Fig. 4.58). Paneth cells were included in the peripheral portion of some of the crypts.

Peyers patches in these sections did not display any obvious changes in structure and were covered by the atrophic epithelium. The peripheral layers of muscle and serosa did not exhibit any notable abnormalities.

Group 2B. Ninety-six Hours:

In most sections examined in this group the villi appeared to be of an optimal height. Shorter villi appeared to be budding from the intestinal glands. In one section a small villus was noted pushing into a cystic space contained within the lamina propria of a larger villus (Fig. 4.59).

Epithelial cells on the tips of the villi tended to be cuboidal in form and goblet cells were rare or absent in these areas (Fig. 4.60). Further down the walls of the villi the cells regained a regular columnar shape and the goblet cell population was re-established. The atrophic villus tip cells were associated with a region of oedema in the lamina propria of the villus tips which contained few cells. There was considerable cell loss from the villus tips (Fig. 4.59). Further peripherally, the lamina propria appeared to be quite normal, although a large number of erythrocytes were present in some of the villi. Duct-like structures in the lamina propria were interpreted as the remnants of degenerated crypts of Lieberkuhn.

Viable crypts were a prominant histological feature, with many of these communicating with the intestinal lumen (Fig. 4.60). Mitotic activity was obvious in most crypts. The level of cell division appeared to be at least as great as that seen in the control animals (Fig. 4.61). There was no oedema around the crypts. Many of the crypts had a dilated lumen but the crypt cells were more typical of this cell type. Although most cells in the crypt exhibited an essentially normal appearance, considerable amounts of pyknotic nuclear debris was noted in and about many crypts (Fig. 4.61).

In one animal in this series a greater proportion of the villus epithelium remained atrophic and the lamina propria contained a more prominant inflammatory cell component. A number of dilated lymphatic vessels were also seen. The crypts in this example had a smaller population of dividing cells and more degenerating cells than the other animals in this series.

Group 2B. One Week:

One week after cessation of the infusion, the gross structure of the jejunal lining appeared similar to that seen in control specimens. Well formed villi were present and most were of optimal height and symmetrical shape (Fig. 4.62). They were covered by a regular, columnar cell lining, giving a smooth outline to the villi. Basophilic nuclei occupied the basal portion of the epithelial cells. A prominant goblet cell population was observed on the villus wall.

Each villus contained a narrow core of lamina propria. The connective tissue population comprised lymphocytes, plasma cells, polymorphs, fibrocytes and endothelial cells in a fine fibrous network. There were no discernable differences between this cell population and that observed in control animals. However, there was some residual oedema under the terminal epithelial cells on a few villi (Fig. 4.63). An occasional dilated lymphatic vessel was noted at the base of the lamina propria, but few lacteals could be identified. The crypts contained viable cells, a number of which were in an active phase of the mitotic cycle. Many of the crypt cells had produced mucous, collections of which filled the lumina of the crypts. Many of the crypts could be

traced to the villus wall and these were of a long cylindrical shape (Fig. 4.64).

The results of the quantified assessment of the jejunal tissues are summarized in Tables 4.3-4.5.

	Time after end of infusion (hours).					
	Control	0	12	24	48	96
Villi	++	++	++	++pr+	+	+or++
Epithelium	++	++	++	++or+	+	+or++
Mitoses in crypts	M	A	A	A	AorP	M
Crypt cell death	A	P	P	P	P	A
Crypt morphology	++	+	+	+	+or-	+or++
Goblet cell number per villus tip	14	10.4	8.3	8.2	8.1	7.7
Lamina propria	++	++or+	++or+	+or-	+or-	+or++

Table 4.3: Group 1. Morphological changes occurring in jejunal tissues following infusion of 10 mg/kg of methotrexate.

Key

++	Normal
+	Abnormal, minor change
-	Abnormal, major change
A	Absent
P	Present
M	Multiple
U	Ulcerated

	Time after end of infusion (hours).			
	Control	0	24	48
Villi	++	+	-	-
Epithelium	++	+	-U	-U
Mitoses in crypts	++	A	A	A
Crypt cell death	P	P	P	P
Crypt morphology	++	+	-	-
Goblet cell number per villus tip	11.7	7.6	rare	A
Lamina propria	++	-	-	-

Table 4.4: Group 2A. Morphological changes occurring in jejunal tissues following infusion of 30 mg/kg of methotrexate followed by 1 ml of normal saline.

Key

++	Normal
+	Abnormal, minor change
-	Abnormal, major change
A	Absent
P	Present
M	Multiple
U	Ulcerated

	Time after end of infusion (hours).			
	0	24	96	1 week
Villus shape	++	+or-	++	++
Epithelium	++	+	+	++or+
Mitoses in crypts	PorA	P	PorM	M
Crypt cell death	P	P	A	A
Crypt morphology	+	+	+or++	++
Goblet cell numbers per villus tip	9.4	8.8	12.0	9.1
Lamina propria	++or+	+or-	+	+

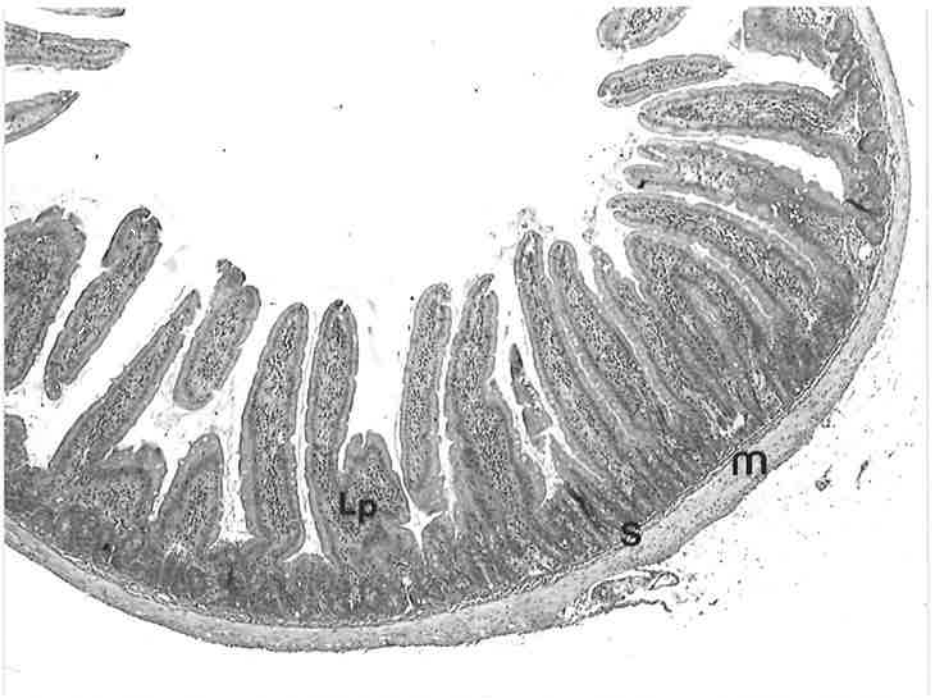
Table 4.5: Group 2B. Morphological changes occurring in jejunal tissues following infusion of 30 mg/kg of methotrexate followed by 30 mg/kg of leucovorin.

Key

++	Normal
+	Abnormal, minor change
-	Abnormal, major change
A	Absent
P	Present
M	Multiple
U	Ulcerated

Figure 4.13: Group 1. Untreated Control.
Note (1) Tall, well formed villi
(2) Muscular layers (M)
(3) Submucosa (S)
(4) Lamina propria (LP)
H & E magnification x 44.

Figure 4.14: Group 1. Untreated Control
Note (1) Crypts of Lieberkuhn (C)
(2) Lamina propria (LP)
(3) Lacteal (La)
(4) Goblet cells (G)
H & E magnification x 220.


Lp
S
m

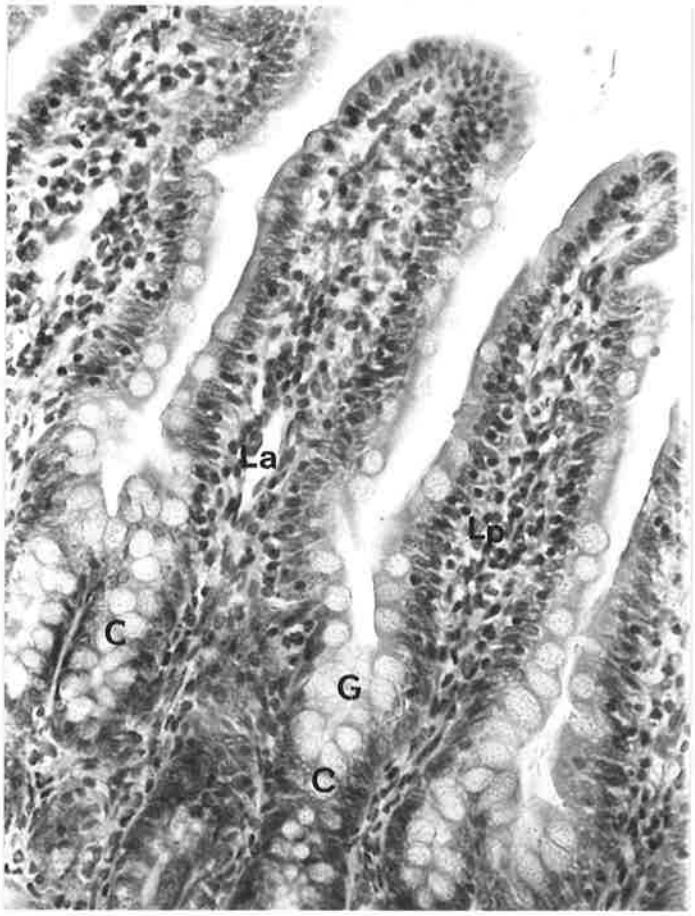
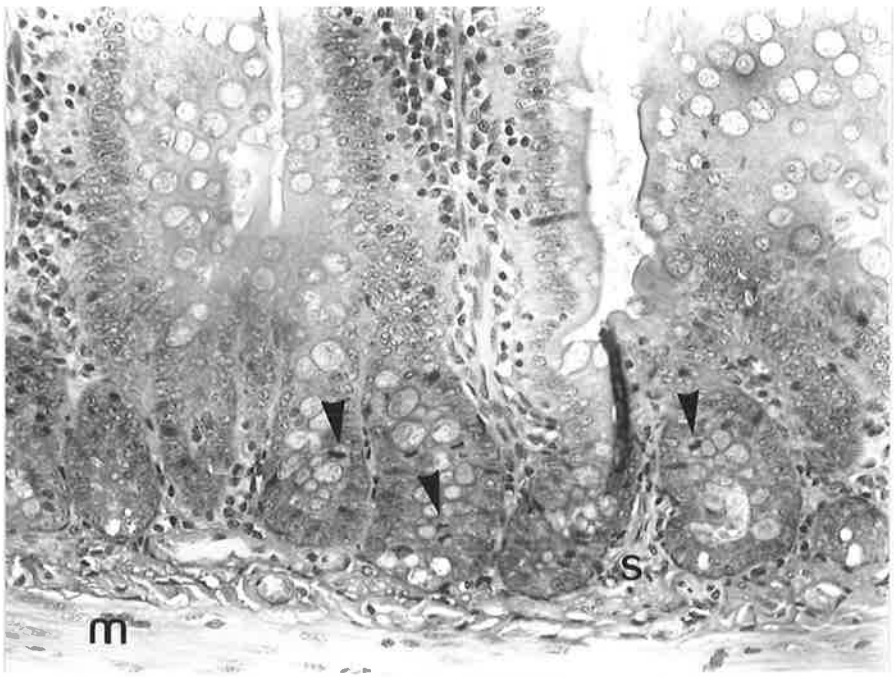
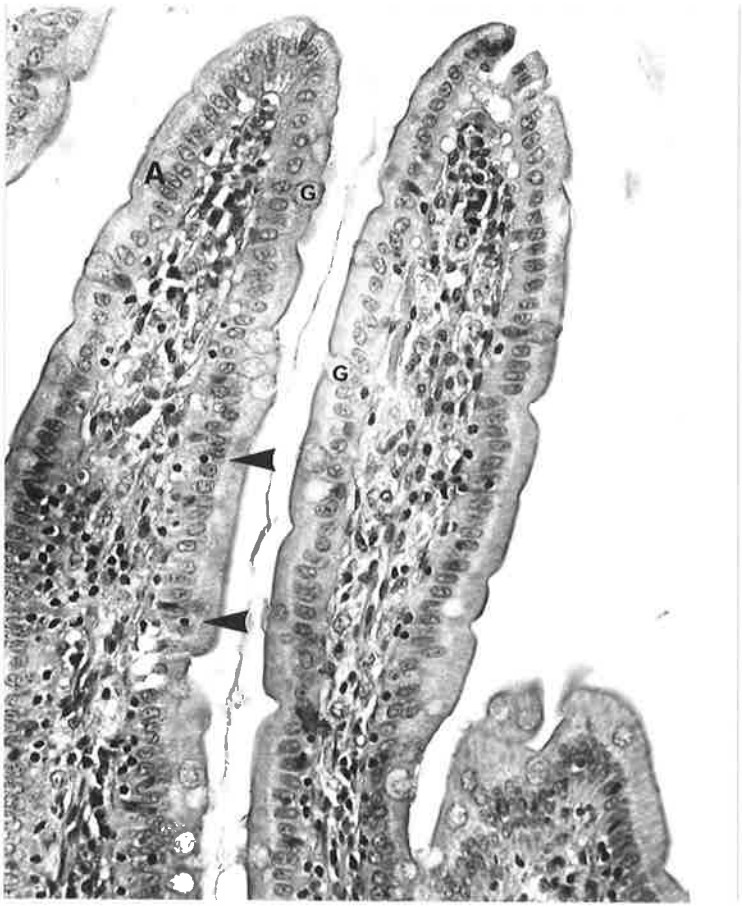

La
Lp
C
G
C

Figure 4.15: Group 1. Untreated Control. Crypts of Lieberkuhn and submucosa.
Note (1) Mitotic figures (arrowheads)
(2) Submucosa (S)
(3) Circular muscle (M)
H & E magnification x 220.

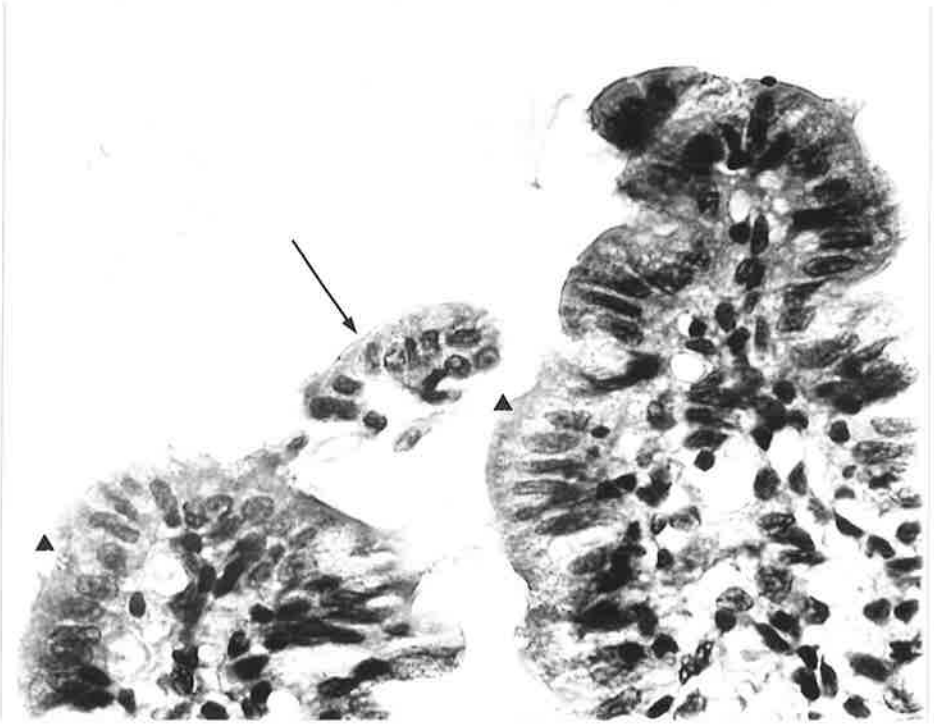
Figure 4.16: Group 1. Untreated Control. Intestinal villi.
Note (1) Regular simple columnar epithelium
(2) Goblet cells (G)
(3) Lymphocytes (arrowheads)
(4) Absortive cells (A)
H & E magnification x 220.


S
m


A
G
G

Fiugre 4.17: Group 1. Untreated Control. Detail of villus tips.
Note (1) Exfoliation of cells (arrow).
(2) Absorbtive cells with ovoid nuclei (arrowheads).
H & E magnification x 440.

Figure 4.18: Group 1. Saline Control.
Note: Well formed villi. Their width is variable owing to the plane of section.
H & E magnification x 44



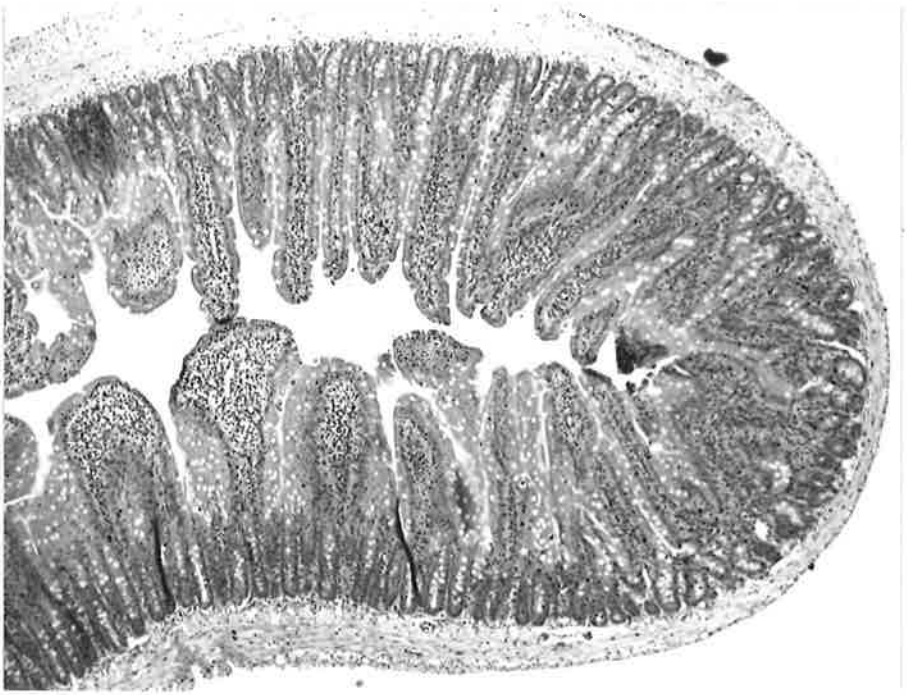


Figure 4.19: Group 1. Saline Control.
Note (1) Tubular crypts of Lieberkuhn (C)
(2) Circular muscle (m)
(3) Longitudinal muscle (M)
H & E magnification x 110.

Figure 4.20: Group 1. 0 Hours.
Note : Well formed villi. These have a regular outline.
H & E magnification x 44.

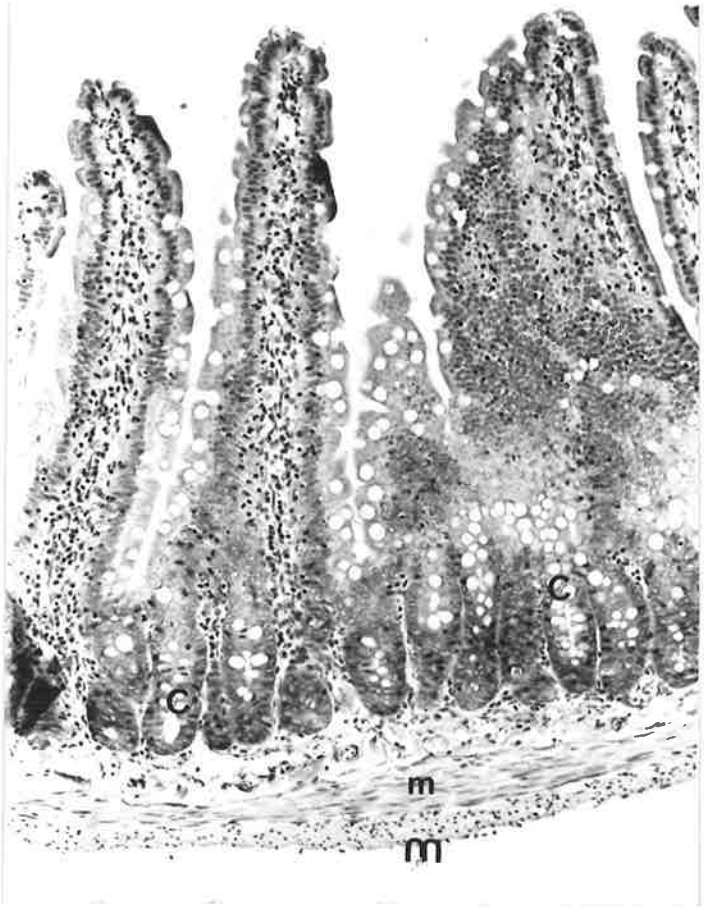
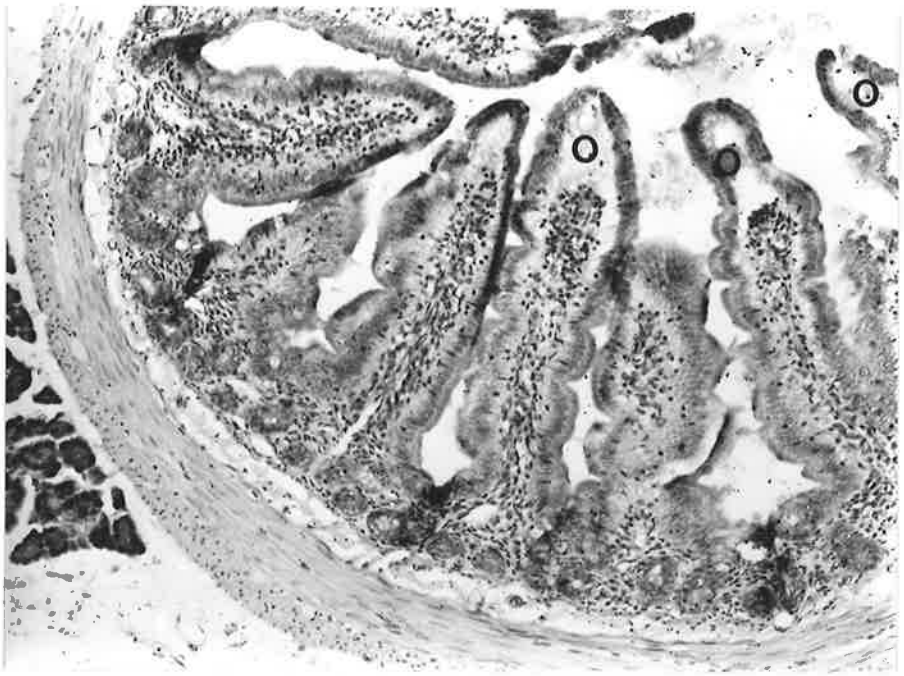

c
c
m
m

Figure 4.21: Group 1. 0 Hours.
Note: Oedema of the villus tips in this section (0).
H & E magnification x 110.

Figure 4.22: Group 1. 0 Hours.
Note (1) Well formed villi with no obvious inflammatory changes in the lamina propria.
(2) Regular epithelial cells.
(3) Intact villus tips.
(4) Ovoid crypts of Lieberkuhn.
H & E magnification x 110.



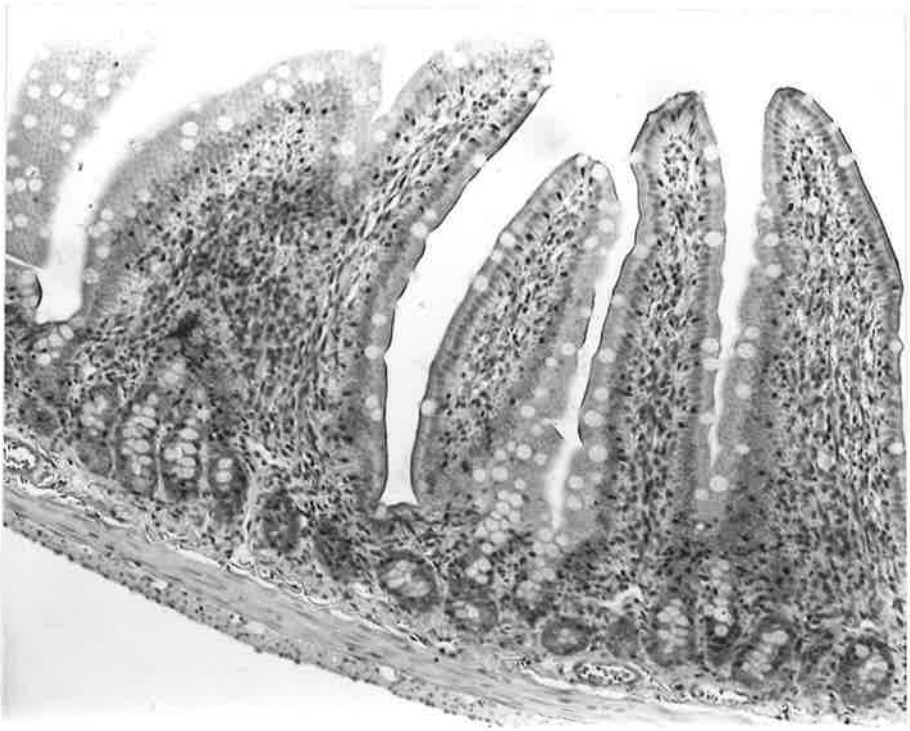
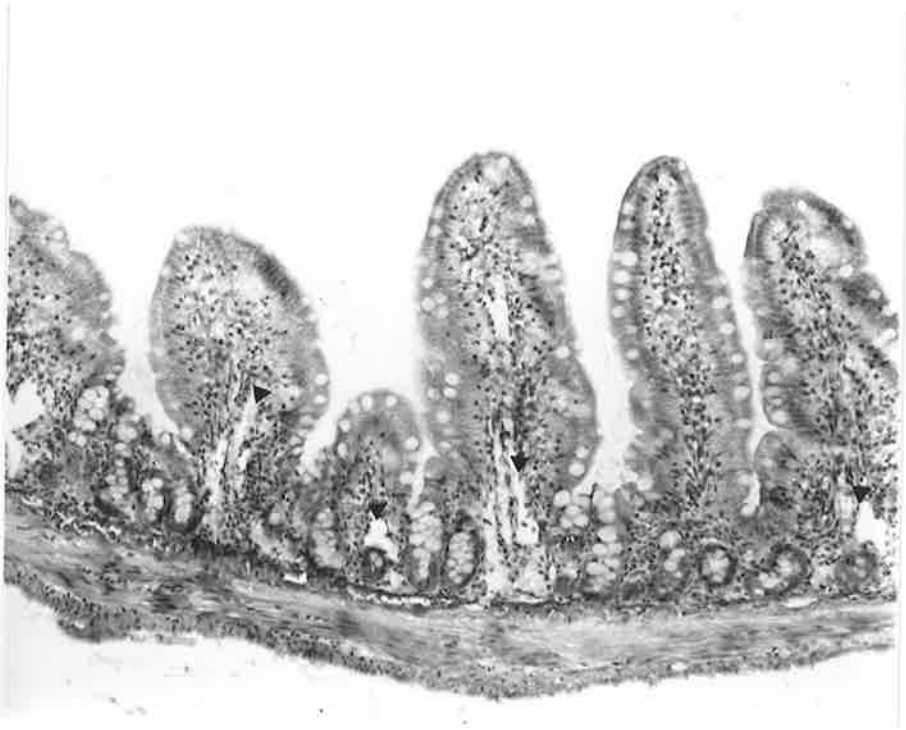


Figure 4.23: Group 1. 0 Hours.
Note (1) Dilated lymphatic vessels in the lamina propria (arrowheads).
(2) Crypts of Lieberkuhn are variable in size and shape.
H & E magnification x 110.

Figure 4.24: Group 1. 0 Hours.
Note (1) Mucous secretions (arrowheads).
(2) Dilated lymphatic vessels (L).
(3) Crypts, of Lieberkuhn are variable in size.
H & E magnification x 220.



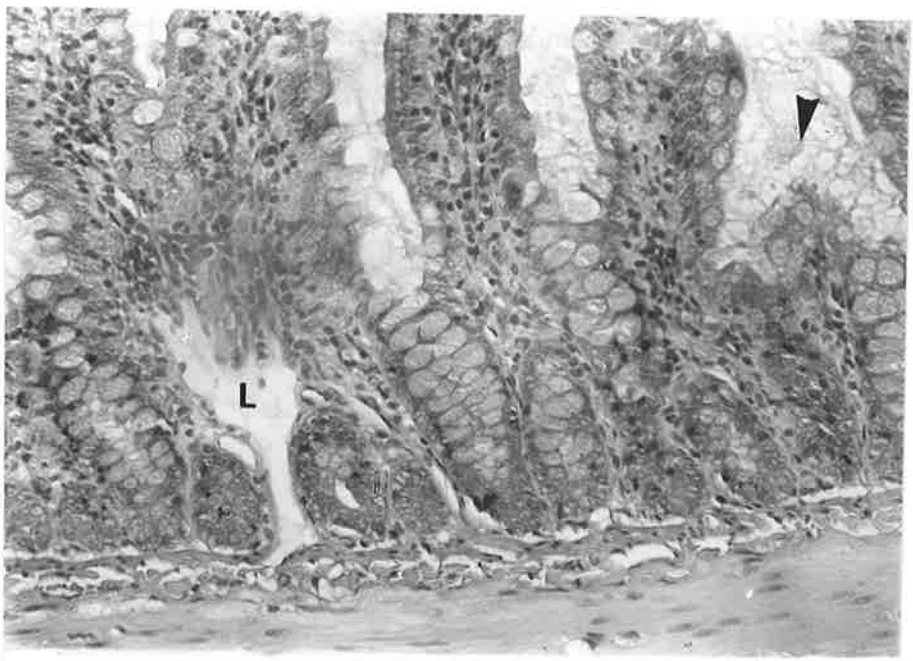
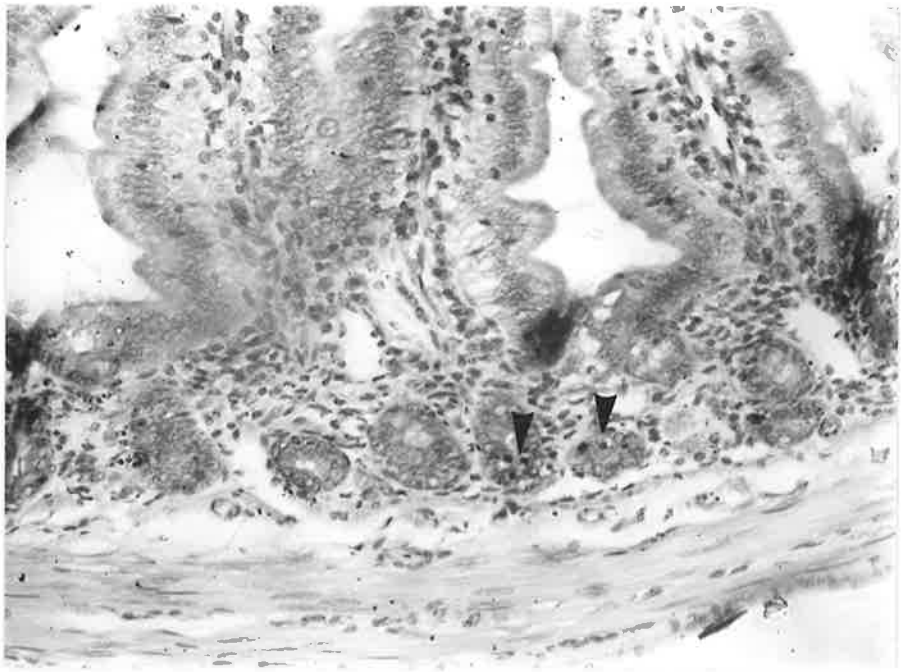

L

Figure 4.25: Group 1. 0 Hours.
Note (1) More severe changes in the crypts of Lieberkuhn. Crypts are decreased in size, are ovoid or circular.
(2) Crypts contain pyknotic nuclei (arrowheads).
H & E magnification x 220.

Figure 4.26: Group 1. 0 Hours.
Note: Karyorrhexis of crypt cells (arrowheads). Mitoses are absent from the crypts.
H & E magnification x 220



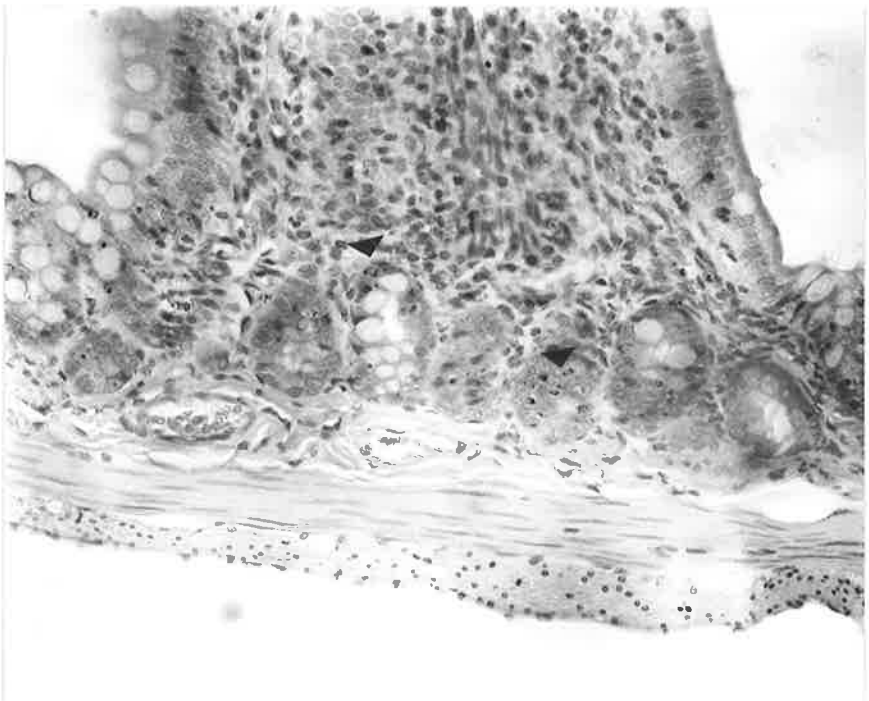


Figure 4.27: Group 1. 12 Hours.
Note: Well formed villi. Tips of villi have been compressed by food in the lumen.
H & E magnification x 44.

Figure 4.28: Group 1. 12 Hours.
Note (1) Villi well formed, but cells are lost from tips
(arrows).
(2) Generalized decrease in crypt size.
(3) Dilated lymphatic vessels at base of villi (L).
H & E magnification x 110.

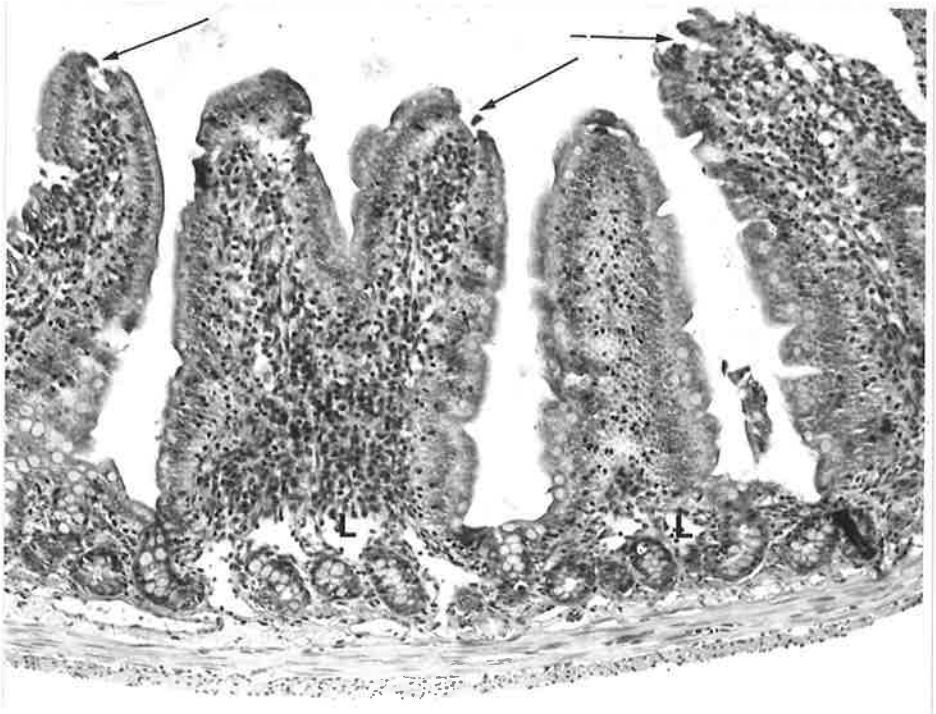
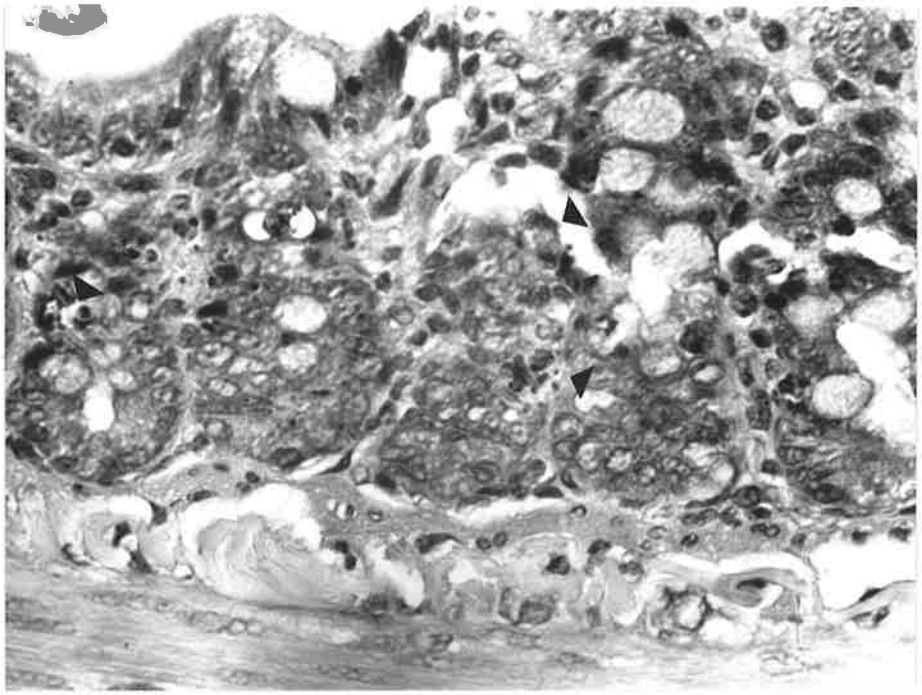


Figure 4.29: Group 1. 12 Hours.
Details of crypts of Lieberkuhn.
Note (1) Mitosis are absent.
(2) Crypt cells degenerating (arrow heads).
(3) Void forming as crypts disintegrate.
H & E magnification x 440.

Figure 4.30: Group 1. 24 Hours.
Note (1) Height of villi appears reduced.
(2) Dilated lymphatic vessels.
Changes are confined to the mucosa.
H & E magnification x 44.



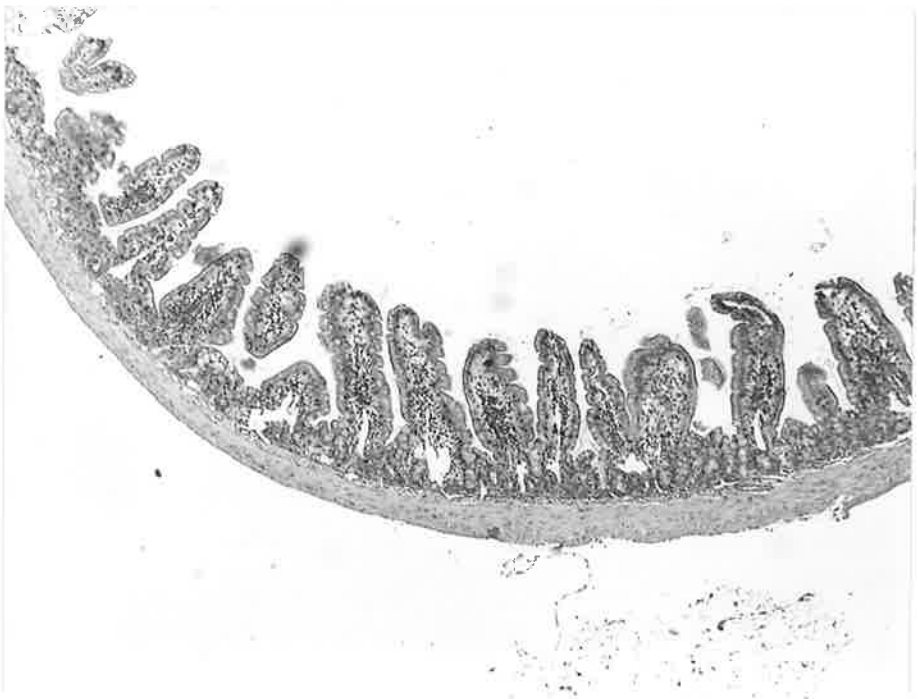
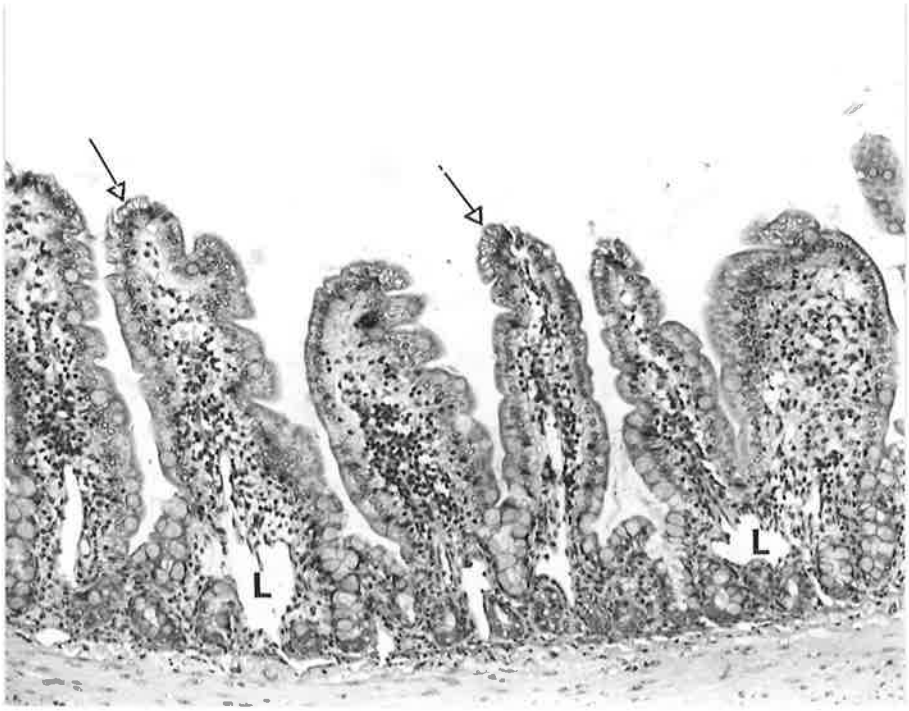


Figure 4.31: Group 1. 24 Hours.
Note (1) Inflammatory cells appear predominant in the lamina propria.
(2) Dilated lymphatic vessels (L).
(3) Terminal epithelial cells display cytoplasmic vacuolation (arrows).
H & E magnification x 110.

Figure 4.32: Group 1. 24 Hours.
Note (1) Flattened irregularly shaped villi.
(2) Clefting in the villis wall (arrows).
(3) Prominant goblet cell population.
H & E magnification x 110.


L
L

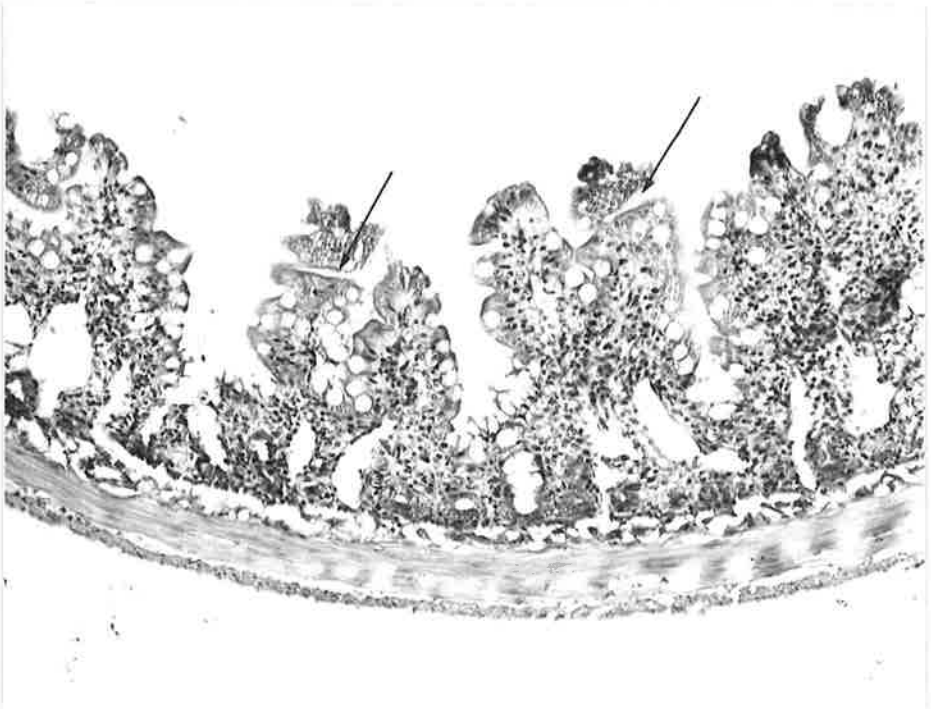
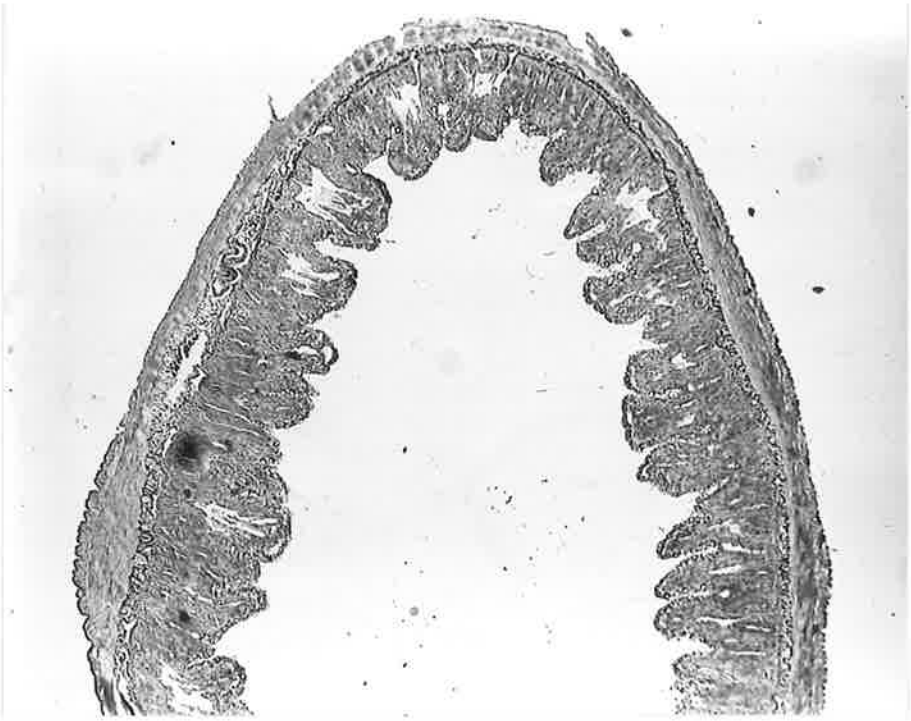


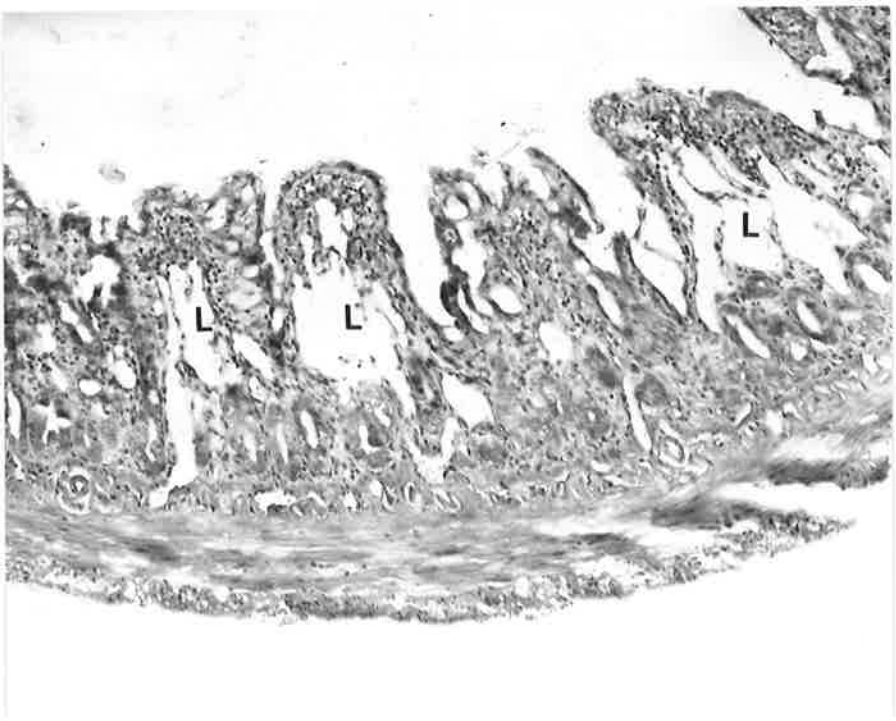
Figure 4.33: Group 1. 24 Hours.
Note (1) Multiple empty goblet cells (M).
(2) Abnormal crypt morphology. Some contain viable cells but are reduced in size (C). Others appear to be undergoing a cystic degeneration (arrow heads).
H & E magnification x 220.


M
M
C
C

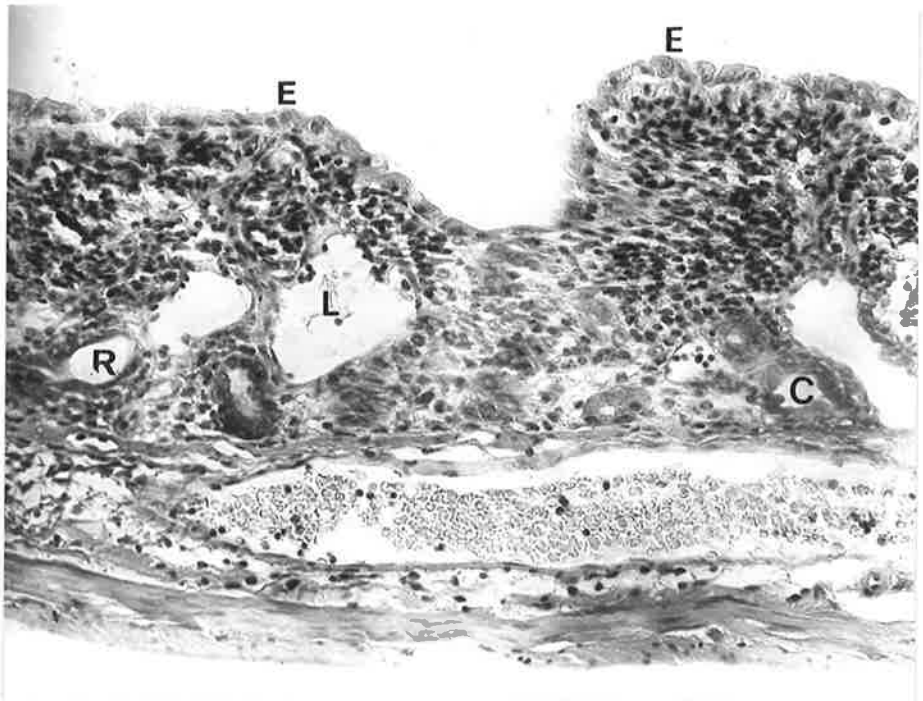
Figure 4.34: Group 1. 48 Hours.
Note: Obvious flattening of villi.
H & E magnification x 44.

Figure 4.35: Group 1. 48 Hours.
Note (1) Flattened, irregularly shaped villi.
(2) Atrophy of the epithelium.
(3) Lymphatic "lakes" in the lamina propria (L).
H & E magnification x 110.




L
L
L

Figures 4.36, 4.37: Group 1. 48 Hours.
Note (1) Grossly flattened villi.
(2) Cuboidal epithelial cells (E).
(3) Dilated lumen of crypts of Lieberkuhn (C).
(4) Cystic remnant of crypt of Lieberkuhn.
(5) Dilated lymphatic (L).
(6) Mitotic figures (arrowhead).
H & E magnification x 220.


E
E
L
R
C

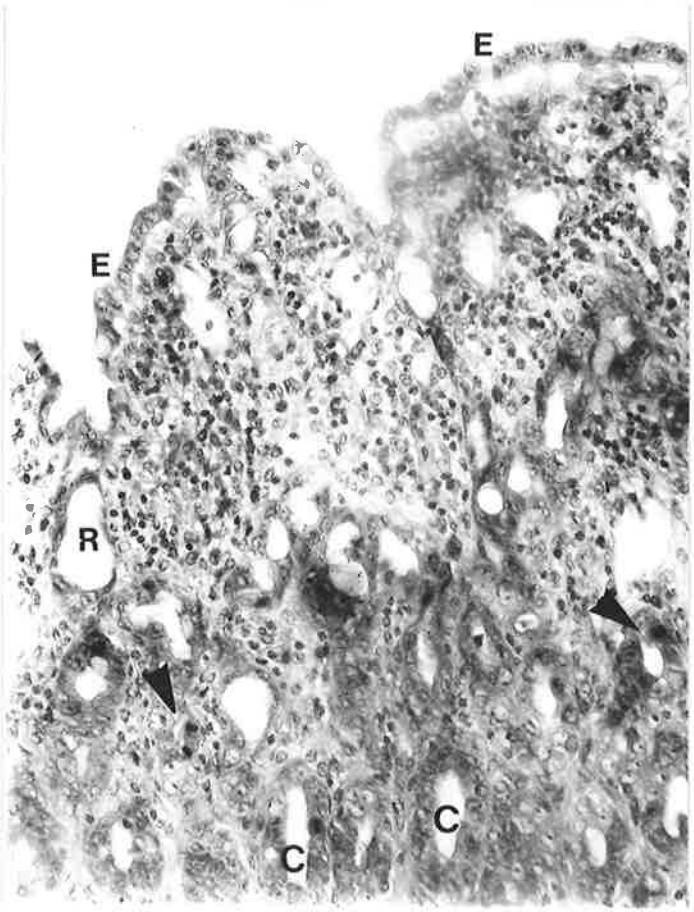
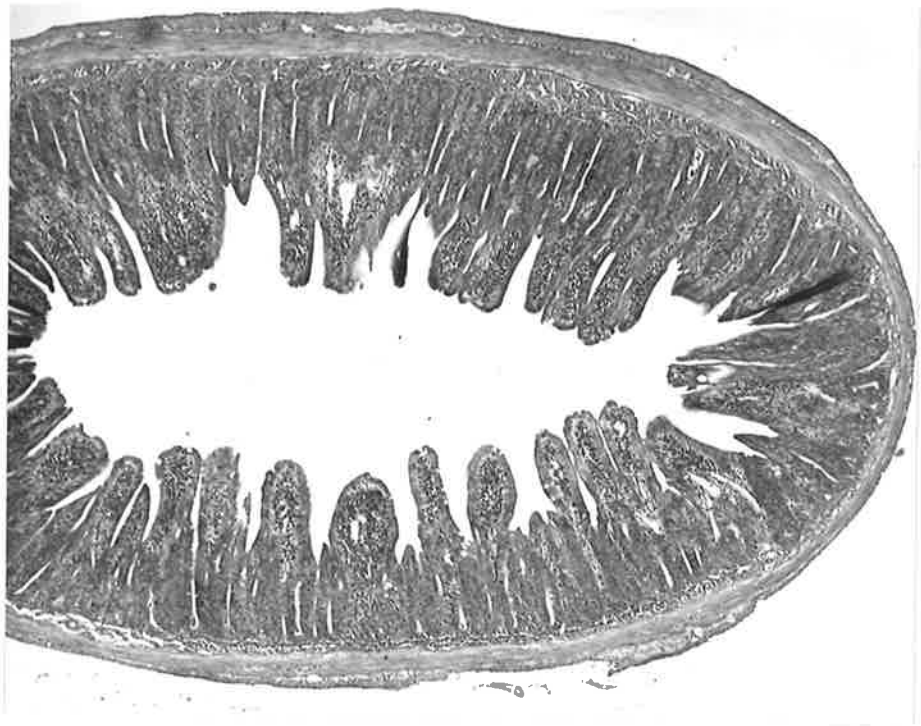

E
E
R
C
C

Figure 4.38: Group 1. 96 Hours.
Note (1) Multiple tall villi with shorter ones intervening.
(2) Deep crypts of Lieberkuhn.
H & E magnification x 44.

Figure 4.39: Group 1. 96 Hours.
Note (1) Villi of good height and intervening short villi.
(2) Crypts of Lieberkuhn are tubular. A number have dilated lumen.
(3) Multiple mitotic figures (arrowheads).
(4) Prominant lacteals (L).
H & E magnification x 110.



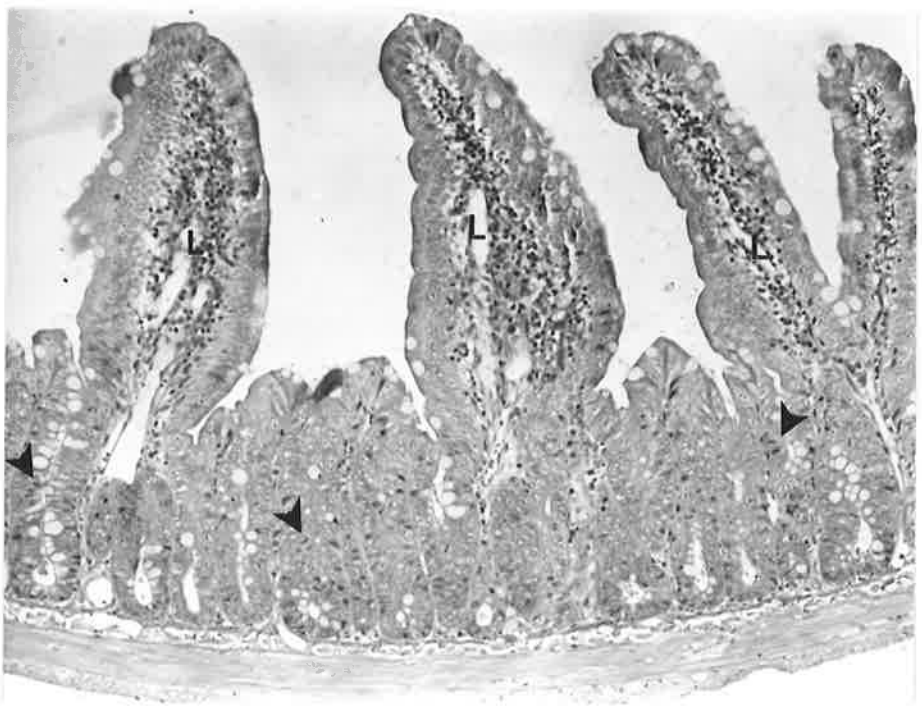
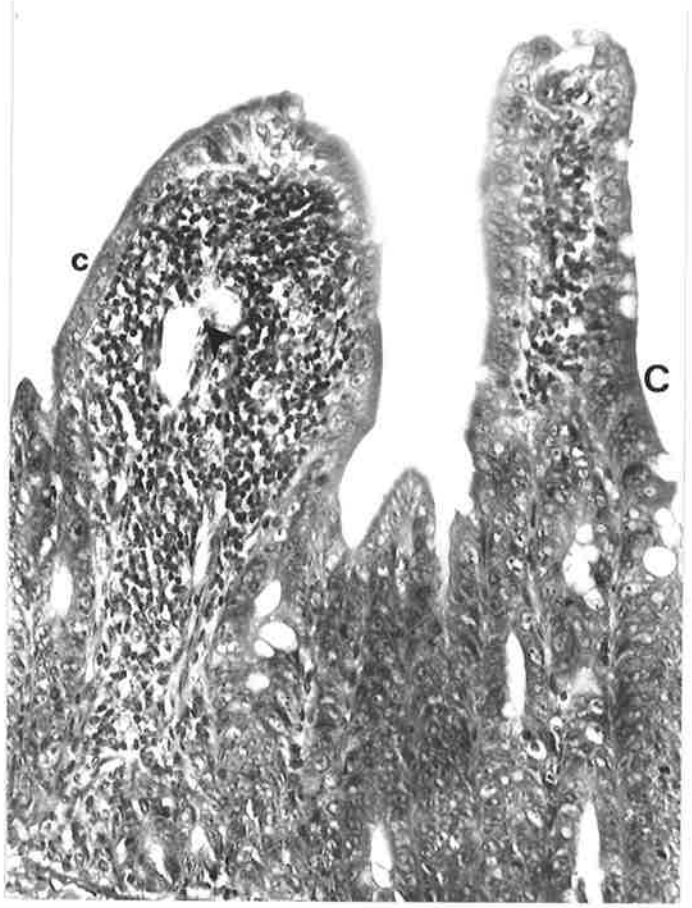

L
L
L

Figure 4.40: Group 1. 96 Hours.
Note (1) Variable epithelial cell covering of villi.
Cuboidal (c), Columnar (C).
(2) Cystic crypt of Lieberkuhn in lamina propria (arrowhead).
H & E magnification x 220.

Figure 4.41: Group 1. 96 Hours.
Detail of crypts of Lieberkuhn. Note two types.
(1) Viable tubular crypts on left side.
(2) Cystic crypts containing cellular debris.
H & E magnification x 220.


c
C

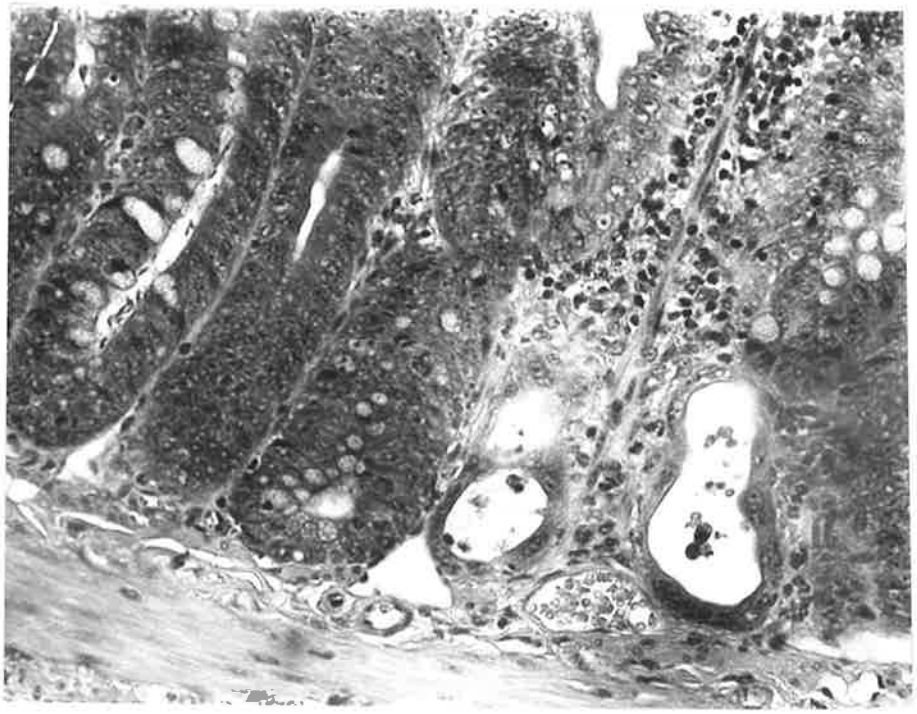
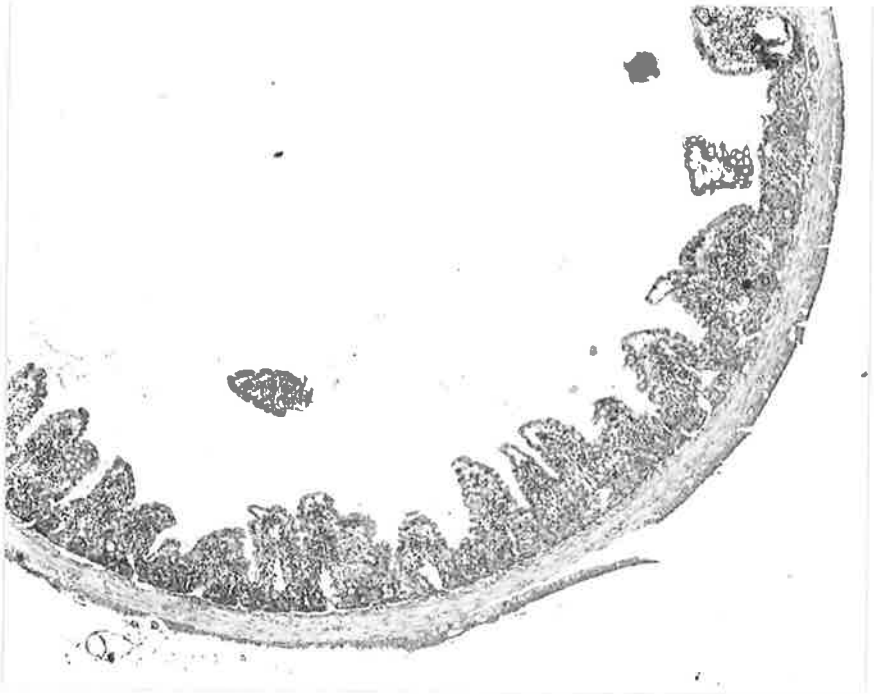


Figure 4.42: Group 2A. 0 Hours.
Villi are reduced in height, flattened and irregular.
H & E magnification x 44.

Figure 4.43: Group 2A. 0 Hours.
Note (1) Reduction in height of villi.
(2) Cuboidal epithelial cells (arrows).
(3) Sub epithelial oedema (O).
(4) Ovoid crypts with dilated lumina.
(5) Site of crypt invaginations now enclosed by a single epithelial cell layer (arrowheads).
H & E magnification x 110.



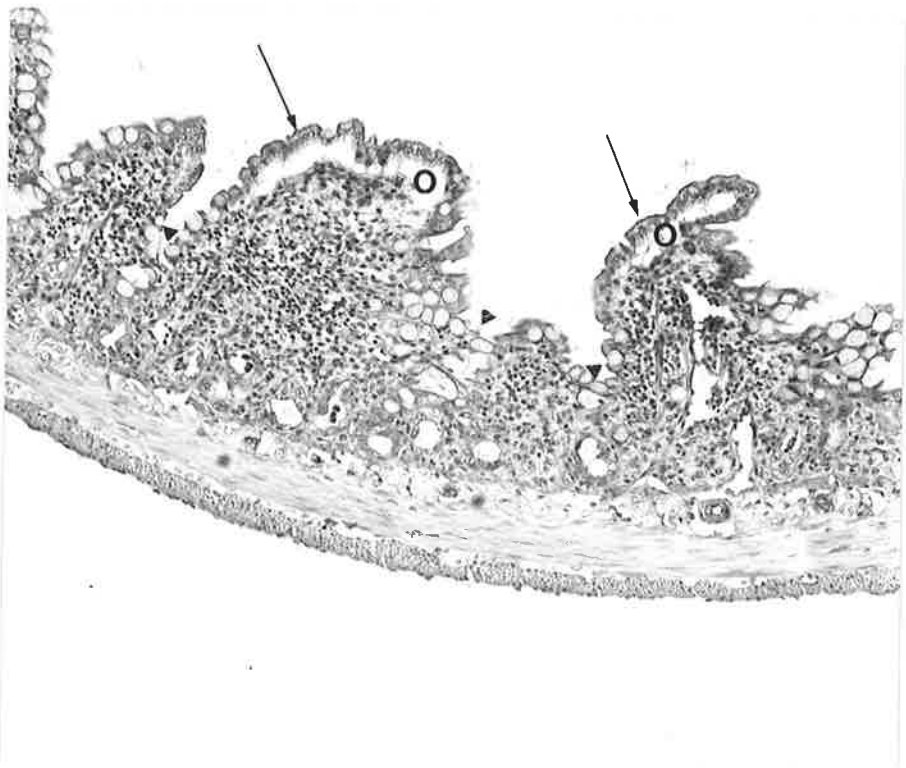


Figure 4.44: Group 2A. 0 Hours.
Note (1) Prominant dilated lymphatic vessels (L).
(2) Exfoliation of epithelial cells (arrows).
(3) Dilation and cystic dgeneration of crypts of Lieberkuhn. These contain cellular remants (arrowheads).
H & E magnification. x 220

Figure 4.45: Group 2A. 24 Hours.
Note (1) Profound flattening of the villi.
(2) Dilation of lymphatic vessels.
H & E magnification x 44.

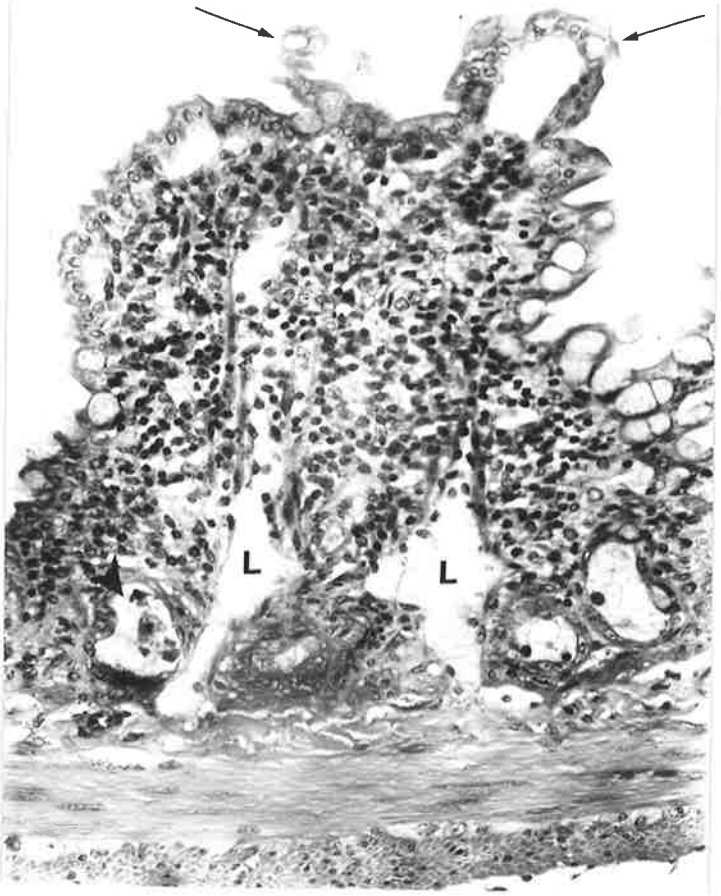
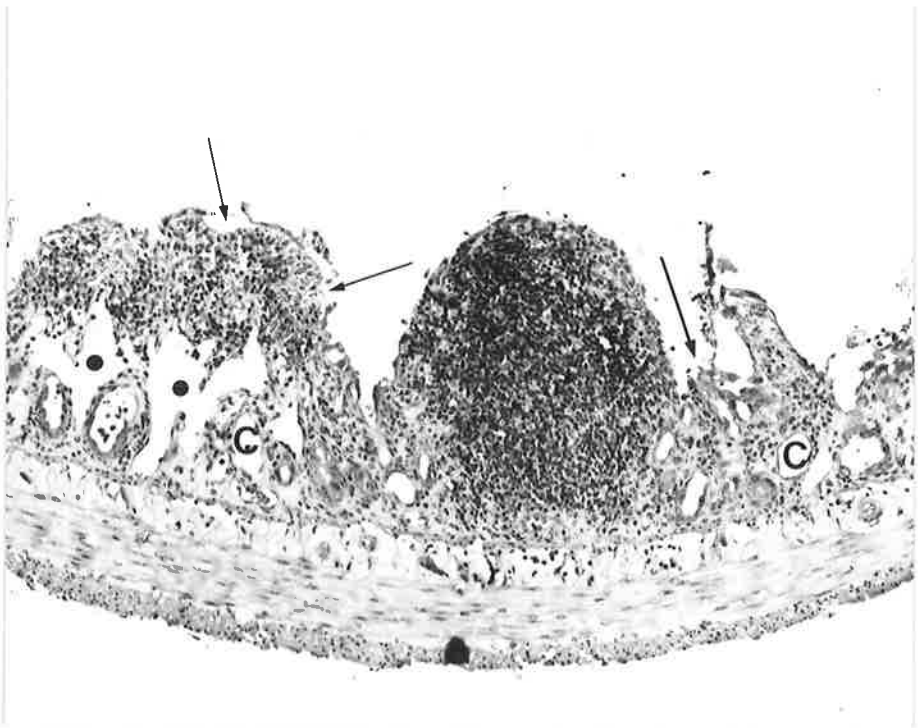

L
L

Figure 4.46: Group 2A. 24 Hours.
Note (1) Flattened villi with fragmentation of epithelial covering leaving microulceration (arrows).
(2) Tortuous dilation and congestion of lymphatics (spot).
(3) Peyers patch with large population of lymphocytes.
(4) Cystic degeneration of crypts of Lieberkuhn (C).
H & E magnification x 110.

Figure 4.47: Group 2A. 24 hours.
Note (1) Gross flattening of epithelial cells.
(2) Fragmentation of epithelium.
(3) Crypts of Lieberkuhn are dilated. Many crypt cells are flattened. Most crypts contain a mixture of dead cells, atrophic cells and normal cells
(4) Cystic degeneration of the crypts may predispose to them coalescing with lymphatic channels (arrow).
H & E magnification x 220.


C
C

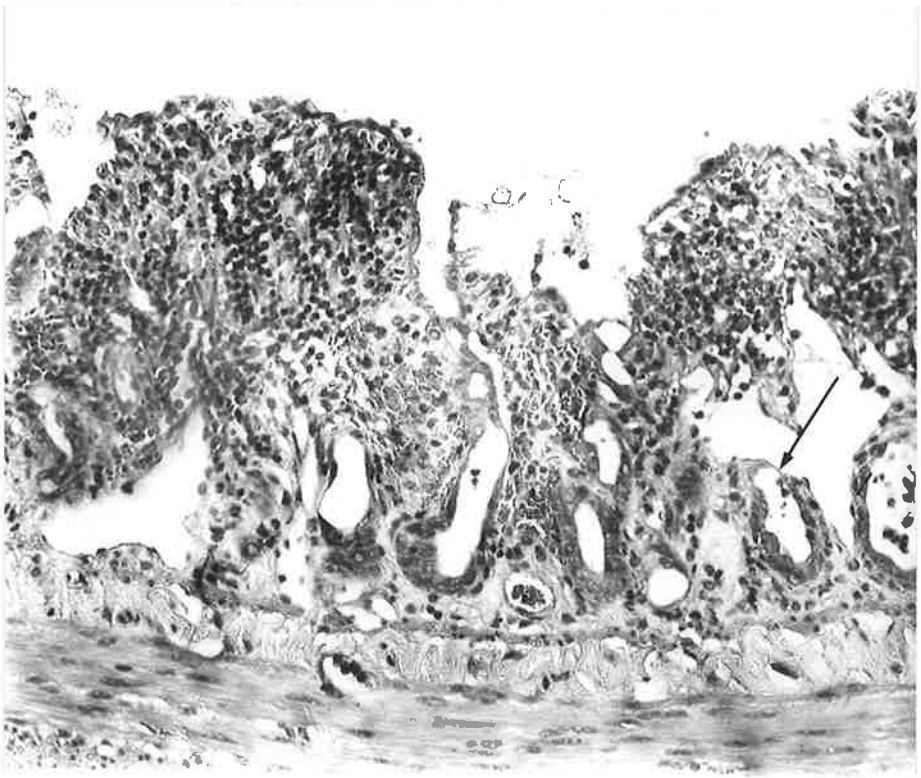


Figure 4.48: Group 2A. 24 Hours.
Detail of epithelium,lamina propria and crypts of Lieberkuhn.
Note (1) Continuous layer of irregular flattened epithelial cells.
(2) Prominant dilated blood vessels (B).
(3) Dilated lacteal (L).
(4) Cystic crypts of Lieberkuhn, flattened cells (arrowheads).
(5) One small crypt with a population of viable cells remains (C).
H & E magnification x 440.

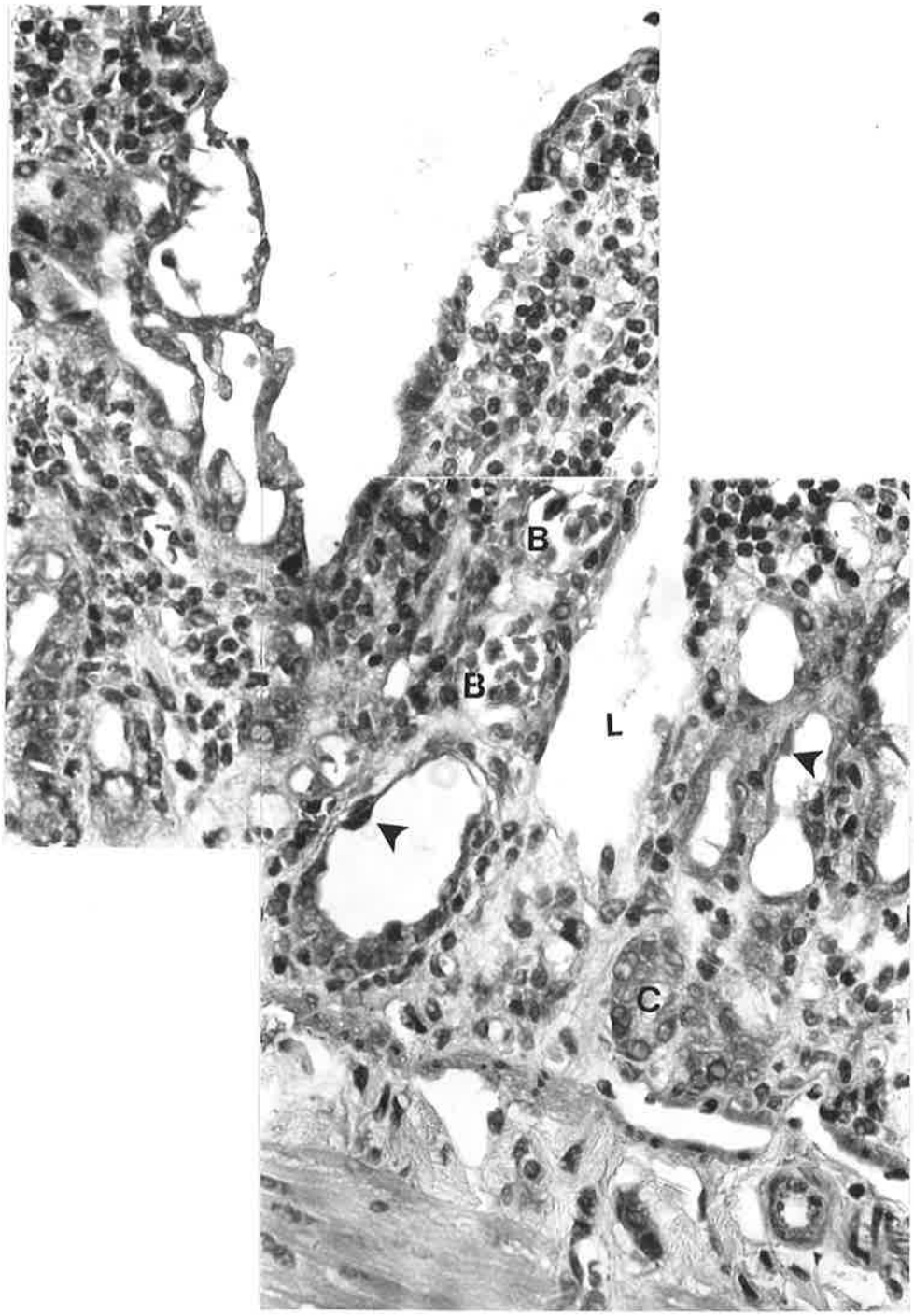
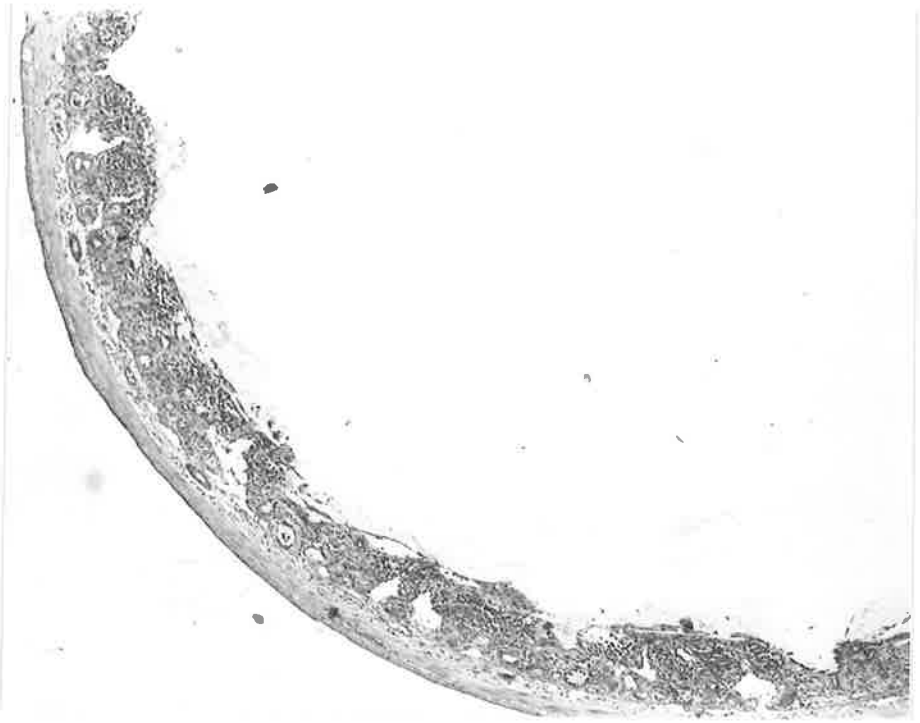
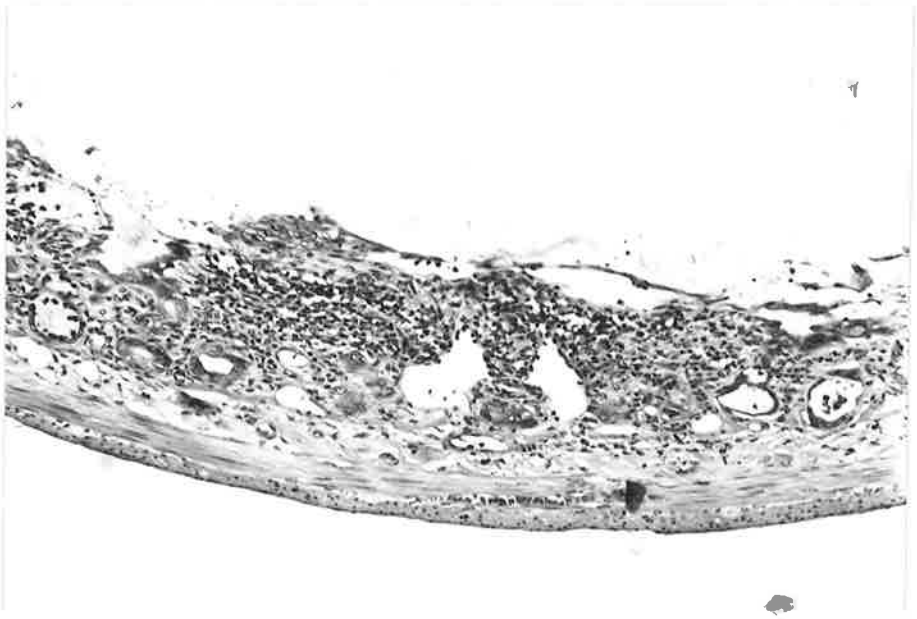

B
B
L
C

Figure 4.49: Group 2A. 48 Hours.
Note: Gross atrophy of the jejunal mucosa.
H & E magnification x 44.

Figure 4.50: Group 2A. 48 Hours.
Note (1) Gross flattening of the villi.
(2) Epithelium fragmenting and lifting from underlying lamina propria.
H & E magnification x 110.





Fiugre 4.51: Group 2A. 48 Hours.
Note (1) Flattened epithelium.
(2) Ulceration (arrowhead).
(3) Microcystic crypts of Lieberkuhn.
H & E magnification x 220.

Figure 4.52: Group 2A. 48 Hours.
Note (1) Normal longitutinal muscle layer and circular muscle layer.
(2) Normal submucosa.
(3) Degenerating crypts of Lieberkuhn containing cell debris (arrows).
H & E magnification x 440.

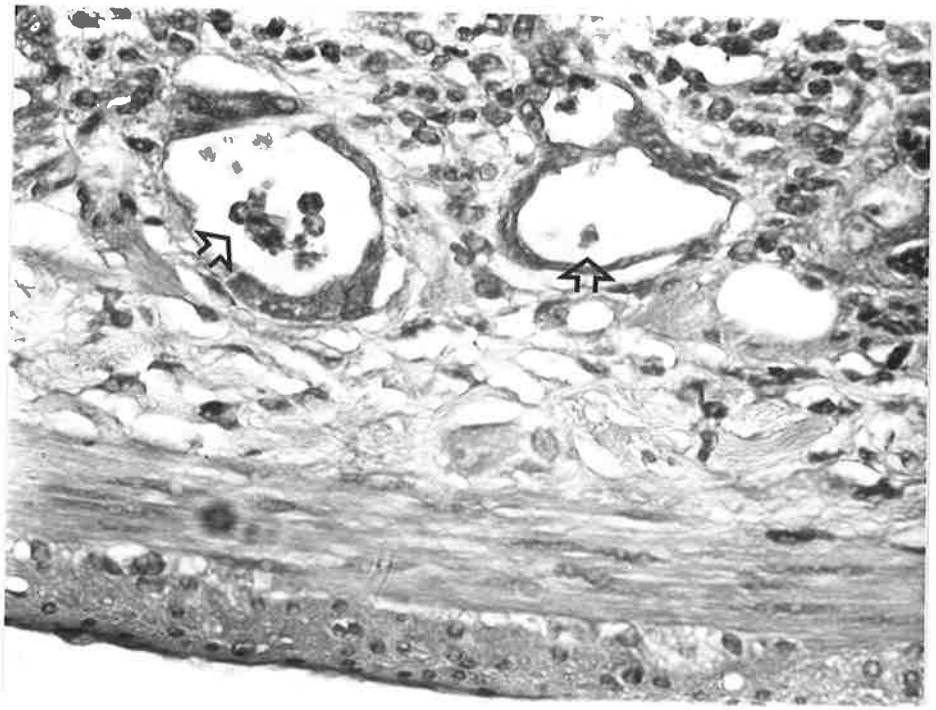
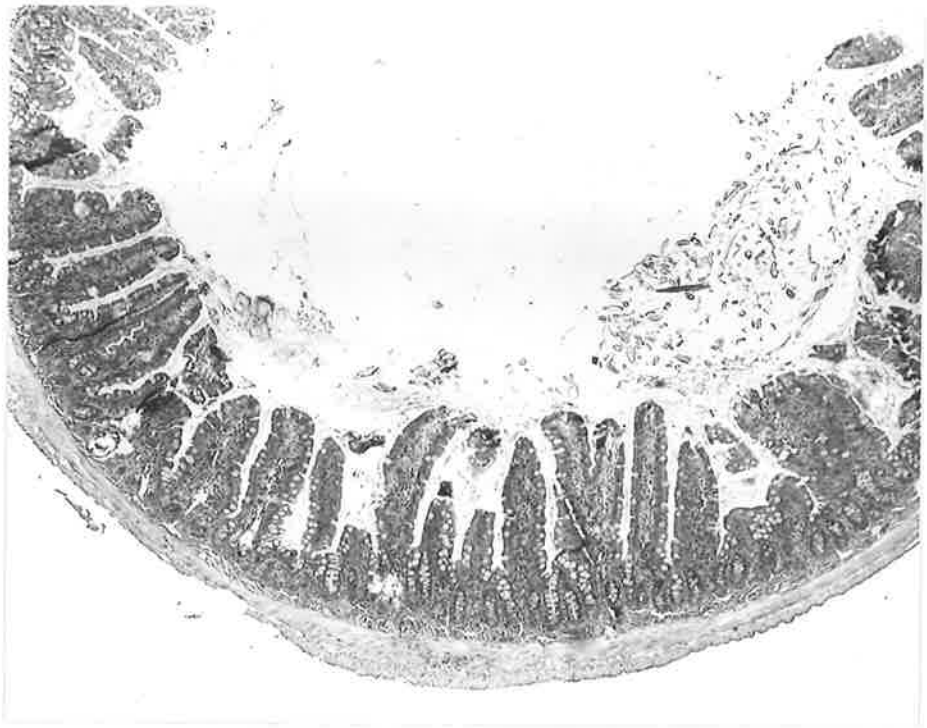


Figure 4.53: Group 2B. 0 Hours.
Note (1) Finger like, orderly villi.
(2) Simple columnar epithelium.
H & E magnification x 44.

Figure 4.54: Group 2B. 0 Hours.
Note (1) Dilated lacteals (L).
(2) Oedema of villus tip (arrow).
(3) Variable shape of crypts.
H & E magnification x 110.



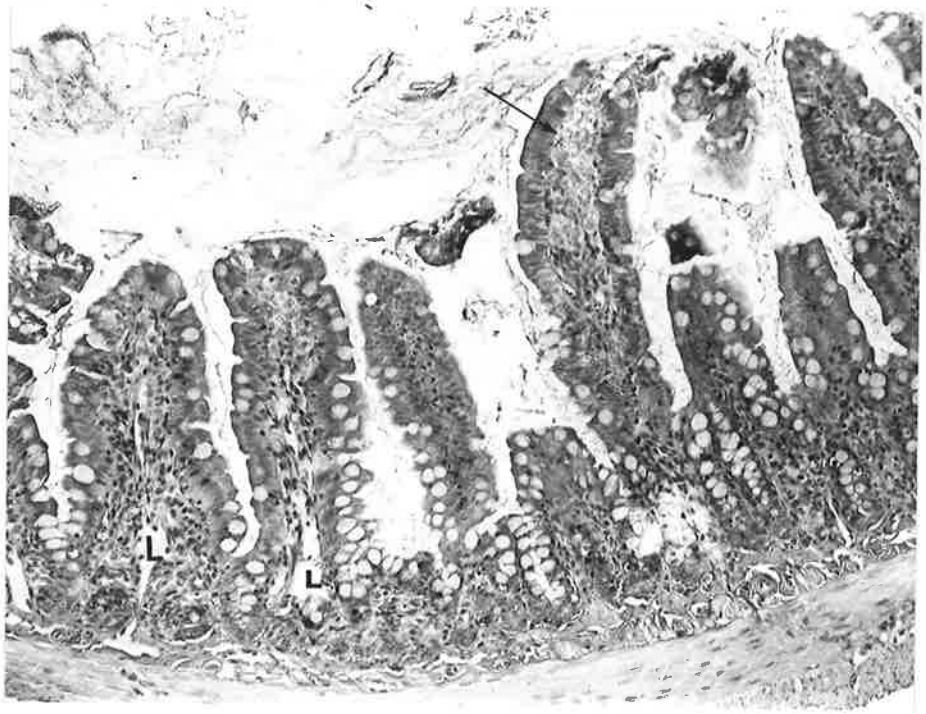
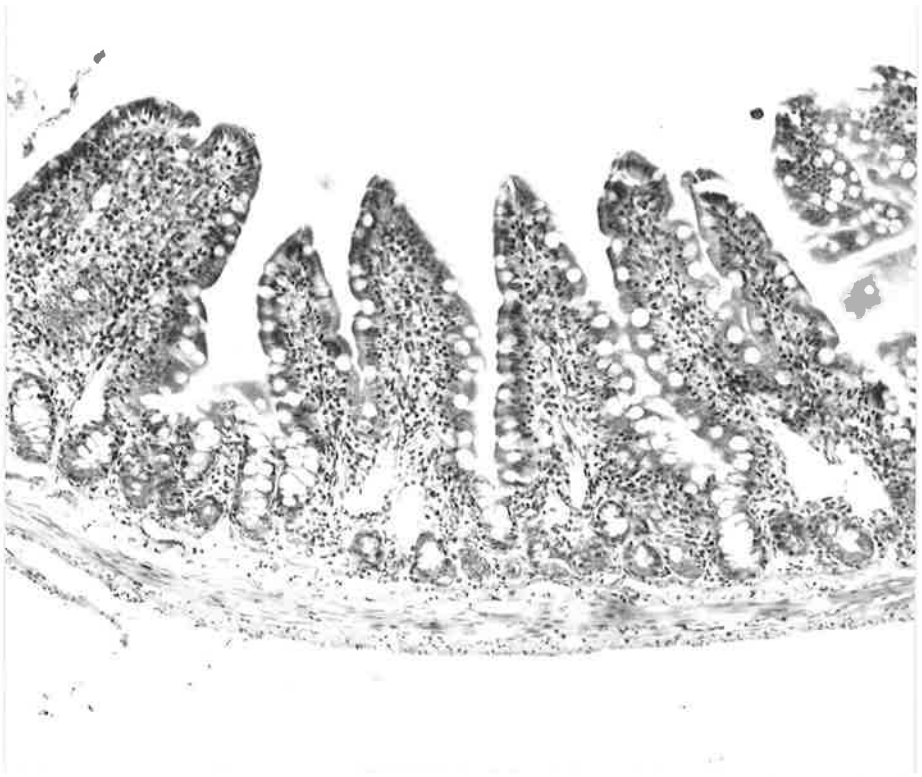

L
L

Figure 4.55: Group 2B. 0 Hours.
Note (1) Preservation of villus shape.
(2) The size of many crypts if reduced.
(3) Tubular crypts contain many goblet cells, smaller crypts contain few.
H & E magnification x 110.

Figure 4.56: Group 2B. 24 Hours.
Note (1) Obvious decrease in height of villi.
(2) Irregular epithelial outline.
(3) Widespread dilation of lymphatics.
(4) Large population of ovoid crypts of Lieberkuhn.
H & E magnification x 44.



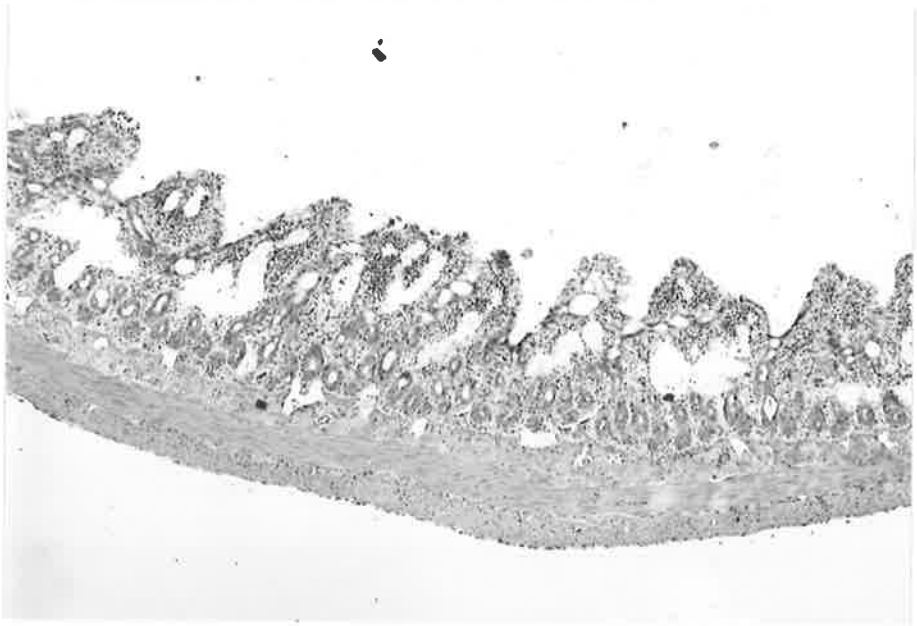
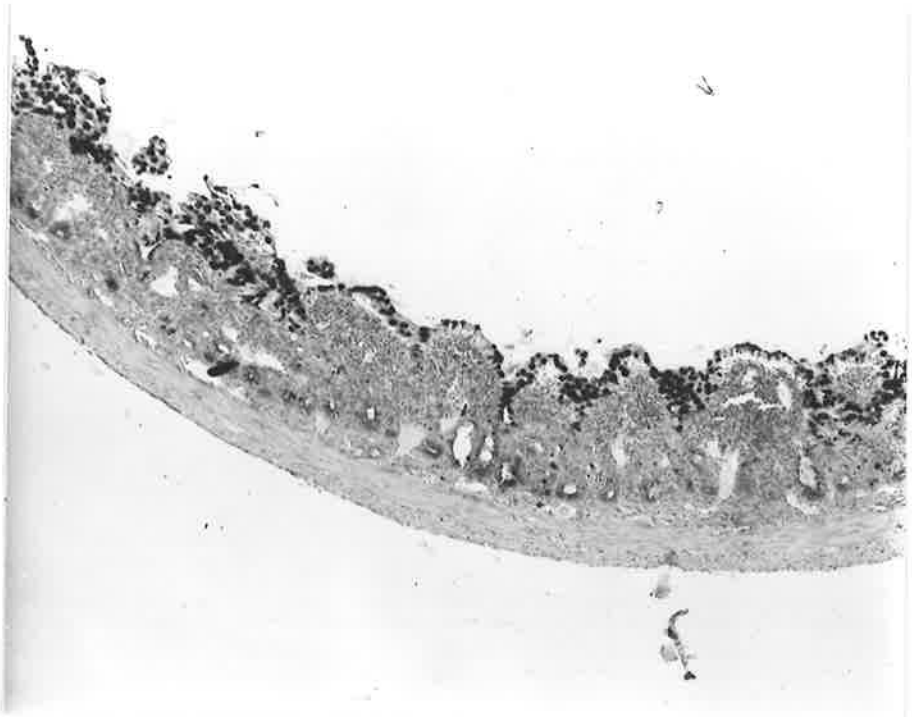


Figure 4.57: Group 2B. 24 Hours.
Note (1) Stain for mucous confirms the continuity of the epithelium.
(2) Goblet cells predominate between the villus tips. Few are present in the crypts of Lieberkuhn.
PAS Diastase magnification x 44.

Figure 4.58: Group 2B. 24 Hours.
Note (1) Cuboidal epithelial cells.
(2) Multiple discharging goblet cells.
(3) Lymphatic vessel (L).
(4) Paneth cells (P).
(5) Mitoses (arrowhead).
H & E magnification x 400.



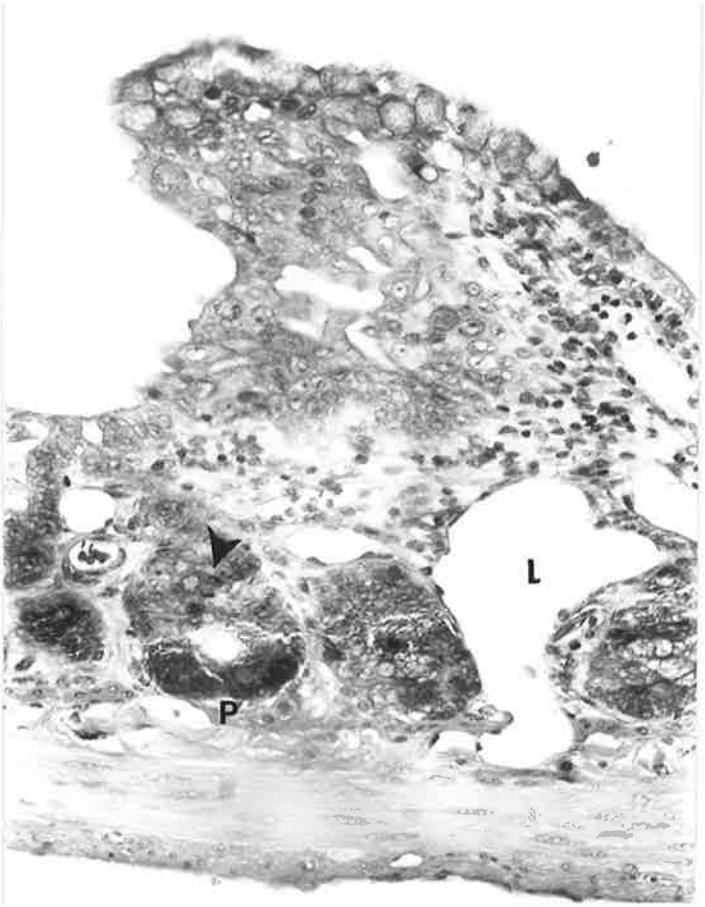

L
P

Figure 4.59: Group 2B. 96 Hours.
Note (1) Well formed villi.
(2) Deep crypts of Lieberkuhn.
(3) Epithelial cell loss from oedematous villus tips.
(4) Small villus growing into cystic space in lamina propria (arrow).
H & E magnification x 44.

Figure 4.60: Group 2B. 96 Hours.
Note (1) Columnar cells on villus walls.
(2) Cuboidal cells on villus tips.
(3) Oedema of villus tips.
H & E magnification x 110.

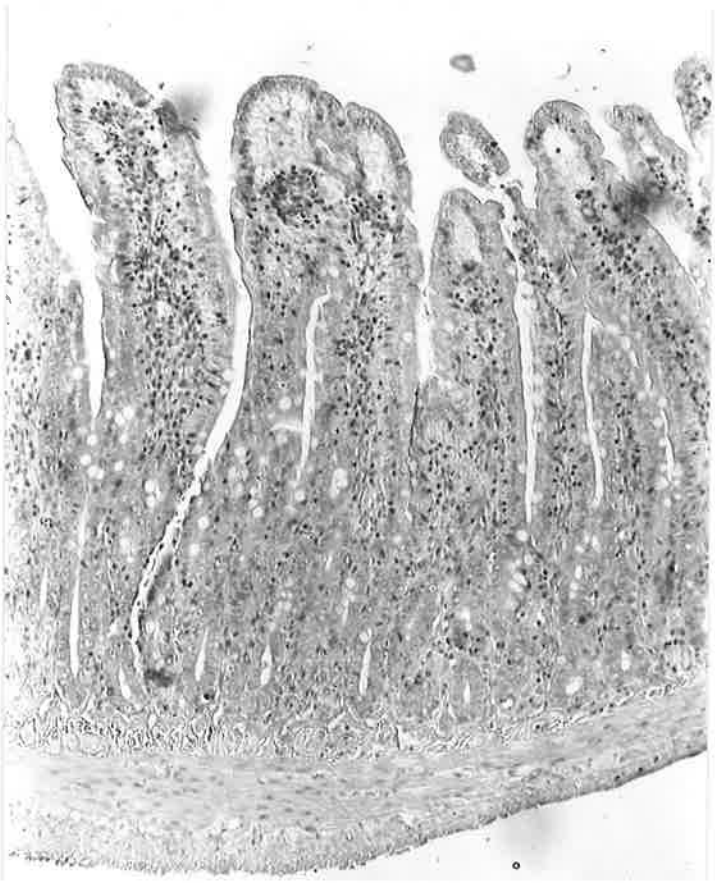
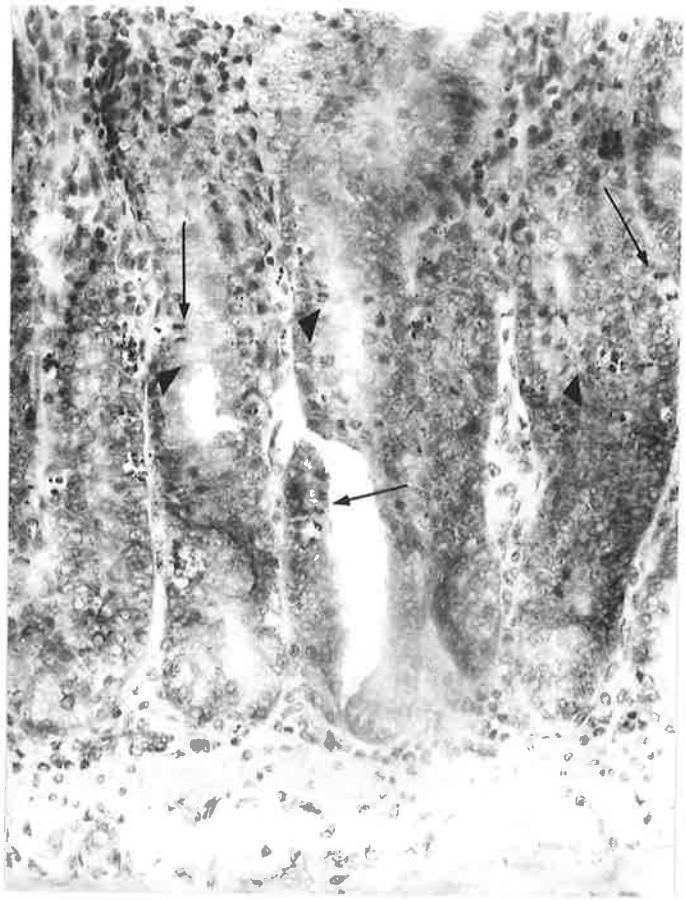


Figure 4.61: Group 2B. 96 Hours.
Note (1) Tubular crypts of Lieberkuhn.
(2) Mitotic figures (arrows).
(3) Nuclear debris scattered throughout tissue (arrowheads).
H & E magnification x 220.

Figure 4.62: Group 2B. 1 Week.
Note: Tall regularly shaped villi.
H & E magnification x 44.



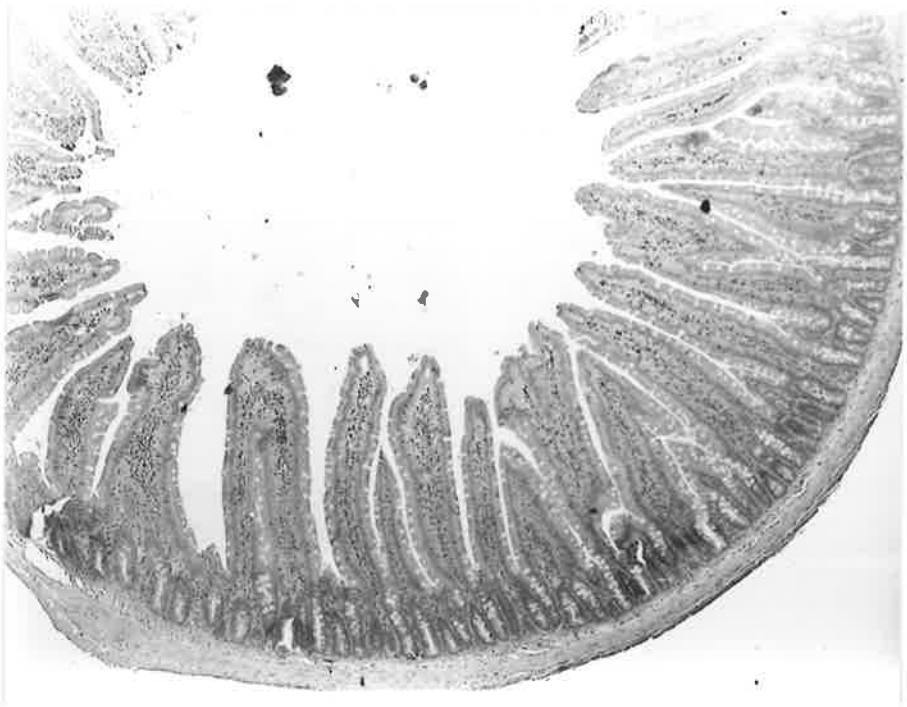
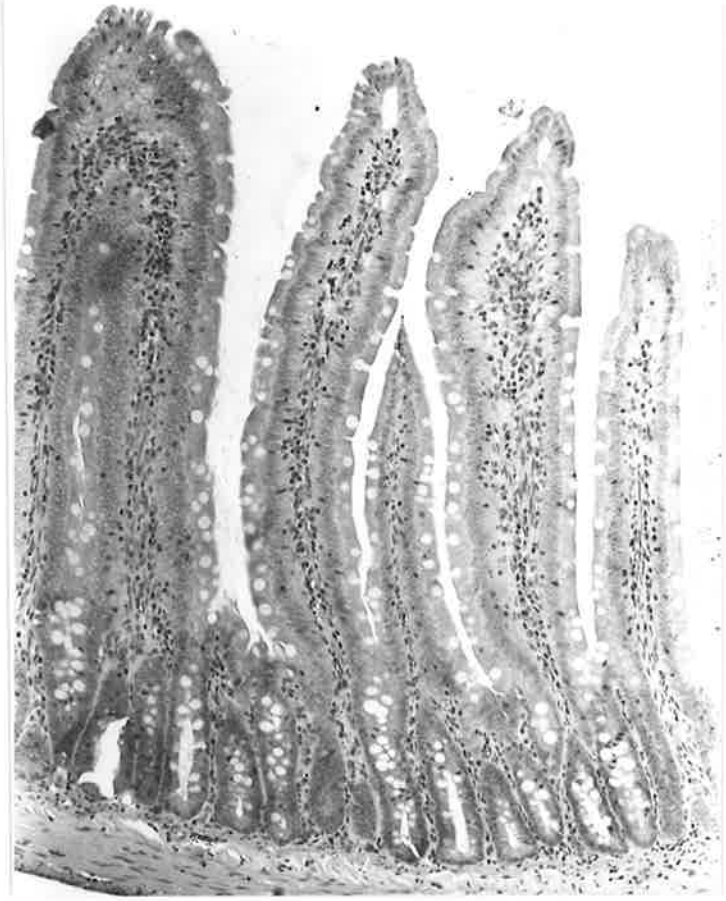
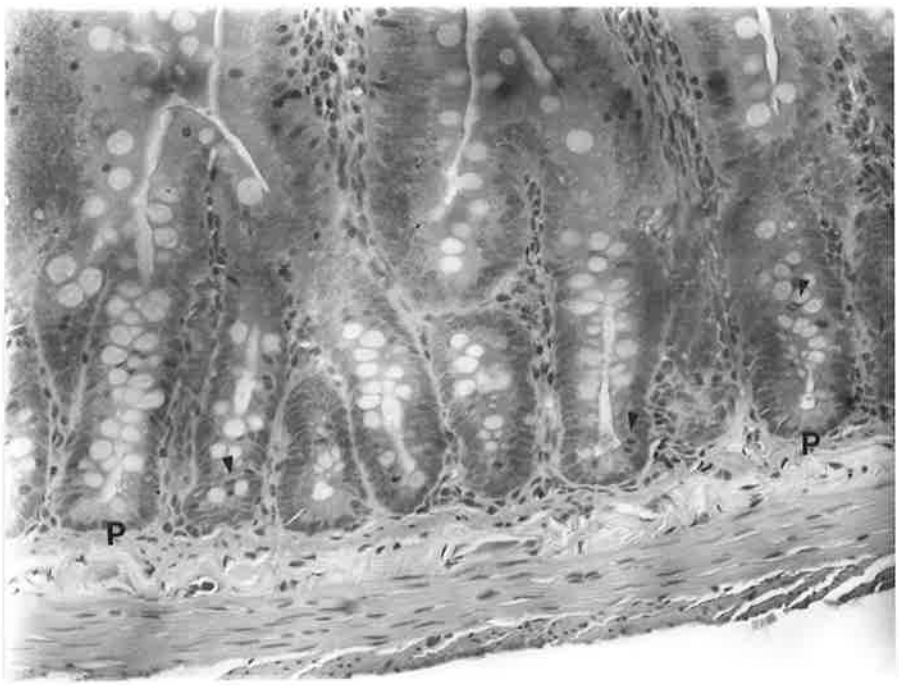


Figure 4.63: Group 2B. 1 Week.
Note (1) Villi covered by simple columnar epithelium.
(2) Tubular crypts of Lieberkuhn.
H & E magnification x 110.

Figure 4.64: Group 2B. 1 Week.
Note (1) Tubular shaped crypts of Lieberkuhn.
(2) Glandular cells with basally orientated nuclei.
(3) Paneth cells at base of crypts (P).
(4) Mitotic figures (arrowheads).
H & E magnification x 220




P
P

CHAPTER 5

DISCUSSION

Porton strain albino rats were chosen as the experimental animal for this study because this strain of rat had previously been used to study the induction of oral carcinoma (Wong 1981, Wong and Wilson 1983) in this department. Further, previous studies of methotrexate in rats carried out by other investigators, provided a guide regarding the toxicity of this agent in rats and its effects on the gastrointestinal tract of these animals. Other factors influencing the decision to use rats were, that facilities were available for housing and operating on the animals, ease of handling and robustness of the animal, and the fact that they are small enough to be easily caged and observed.

Design of Experiments

As was discussed in the review of the literature, induced or transplanted solid neoplasms in rats tend to be resistant to chemotherapy. As it could be expected that induced oral squamous cell carcinomas would share this property, some care was required in the design of a drug regime in this study, which would have the maximum chance of causing a clinical and histological response in these neoplasms. In this context the toxicity of the drug schedule had to be sublethal to allow the animals to survive. This would allow the response of tumours in any future studies to be assessed over several weeks, and allow the effects of a second pulse of the drug to be studied. Long-term antitumour therapy such as daily single doses with this drug, permit only a very small dose to be given before severe toxicity becomes a

problem. Such low doses would not be expected to affect a potentially resistant, solid neoplasm such as an oral squamous cell carcinoma.

The literature reveals that successful chemotherapy of solid, transplanted rat neoplasms can occur when high (but sublethal) doses of drug are used, particularly by intermittent infusion. This method of drug administration is also the usual method of administration when delivered in high doses to humans. For the present study therefore, it was considered that methotrexate should be delivered to the rats in relatively high doses by an infusion technique. It was theorized that this would provide high serum levels of the drug (able to penetrate a solid neoplasm) over a number of hours, allowing a large number of malignant cells to become mitotically active and thus susceptible to the drug. If necessary, the drug could then be withheld until the recovery of normal tissues, but readministered, allowing the monitoring of the effects of a second drug challenge.

Zaharko and Dedrick (1973) showed that the small intestine of rodents will tolerate a complete interruption of DNA synthesis for approximately 24 hours. Therefore, it was decided that the infusion in the present experiment should continue for only that period.

The dose of methotrexate to be delivered was set initially at the LD 10 level. This dose has significant toxicity but allows the survival of 90% of animals. Freireich et al (1966) have shown that the LD 10 level in animals correlates well with the maximum tolerated dose in humans. The literature describing both human and animal trials supports the view that solid tumours tend to respond better to higher dose regimes of methotrexate. In order that patients and animals survive high

doses of drug, it is necessary to use a rescue agent, ideally leucovorin. In designing the present experiments it was felt that a higher dose schedule, with rescue, could be compared with the schedule without rescue if the toxicity of the former was in the order of the LD 10. Thus, an arbitrary decision was taken to attempt to rescue the animals from the estimated LD 90 drug dose. This could be expected to be at least twice the LD 10 dose judging from the data of Ferguson et al (1950).

Various routes of drug administration were considered. In man methotrexate has been delivered orally, intra-arterially, intravenously, intraperitoneally, intrathecally and intramuscularly. In experimental animals all of these routes, plus the subcutaneous route, have been used by various researchers (Table 1.2). Since the present study was designed as a precursor to subsequent experiments in tumour bearing animals, it was considered that intra-arterial administration would offer few benefits over an intravenous route, as oral carcinomas are not isolated on a regionally perfusable limb or organ. An intravenous route is attractive for such studies as it provides reliable levels and distribution of the drug. However, while it is not terribly difficult to insert an intravenous cannula into a rat vein, problems are likely to arise when it must remain in situ for a long period. The catheter may become clogged with a blood clot or be dislodged. For these reasons, intraperitoneal was chosen as the route of drug delivery in this investigation. This route has several advantages. Its use is technically undemanding. One can ensure that the catheter is in place and unlikely to be dislodged. The absorption of drug is reliable from the peritoneum and in rodents, toxicity of methotrexate from the intravenous and intraperitoneal routes is usually comparable (Freireich et al 1966).

Although the anaesthetic agent was also delivered by the intraperitoneal route, these agents are not known to interact.

Consideration had to be given to the dose of the rescue agent to be administered, the duration of the rescue regime and the time of commencement of the rescue. Esminger et al (1979) stated that irreversible damage is done to mouse small intestinal mucosa if mitotic activity is interrupted for more than 24 hours. Goldin et al (1954, 1955) confirmed the importance of the timing of rescue when they found that the effectiveness of leucovorin as a rescue agent could not be improved by an increase in its dose, if administration was delayed for 48 hours. Maximum antitumour effect occurred when rescue was commenced 12 hours after aminopterin had been given. Assuming that the rat small intestine behaves similarly to that of the mouse, it was felt that toxicity could be minimized in this experiment by commencing rescue immediately following the methotrexate infusion period, as it could be expected that DNA synthesis would have ceased soon after the loading dose had been injected. This is consistent with the work of Sirotnak et al (1977) who observed the cessation of DNA synthesis in the small intestine of rodents within two hours of the injection of methotrexate.

In clinical circumstances, the dose of leucovorin needs to be sufficient to rescue normal cells, but small enough to minimize the chance of rescue of malignant cells. If serum methotrexate levels are greater than 10^{-5} M in certain animal cell types, up to 100 times the equimolar dose of leucovorin must be given in order to afford protection from the toxic effects (Pinedo et al 1976). If serum levels are 10^{-6} M, equimolar doses of leucovorin are likely to be adequate. Sindram et al (1974) delivered leucovorin at 4 times the daily methotrexate dose and

increased the LD 50 in their experimental rats from 0.21 mg/kg/day to 0.56 mg/kg/day. However, this dose also rescued the malignant cells as tumour growth was greater than with methotrexate given alone. Esminger et al (1979) reported a maximum kill of leukaemic cells in mice when daily leucovorin rescue was kept to levels of 20% of the methotrexate infusion immediately preceeding the rescue. To minimize toxicity the rescue agent would need to be delivered until the serum methotrexate level fell to the threshold concentration of toxicity, which in mouse small intestine is 5×10^{-9} M (Pinedo et al 1977). Because leucovorin has a short half-life it is necessary to deliver it by incremental injection or, by infusion. In the current investigation it was convenient to use intraperitoneal infusion.

Two types of devices are available for drug infusion in experimental animals. The first is the external infusion pump, of which there are many designs. The second is the implantable "mini" pump. Use of the latter has been reported by Pinedo, Zaharko and Dedrick (1976) and Dedrick et al (1974). They typically deliver 0.5-10 uml/hour over 7-28 days. While these devices are attractive, they are unable to deliver the drug doses in the time required for the present experiments. Because of this a decision was made to use an external infusion pump. This style of drug delivery is relible, but the experiment must be designed to ensure that the animal is unable to interfere with the apparatus. Intricate devices have been described which allow the rat to move, despite the presence of an indwelling catheter. Eve and Robinson (1963) designed a swivel conduit to allow free movement of a rat about a special cage while an infusion was given for several days. In another system, Jones and Hynd (1981) placed a wire reinforced tubing over the animals tail and led the infusion catheter out of the cage, thus

allowing the rat limited movement. Facilities were not available in the present study which would allow the use of complex swivelled connections since only routine cages were available for housing the rats. Waynforth (1980) discussed the use of the Bollman cage, which is suitable for short periods of restraint of rats up to several days. Waynforth reported that if animals are allowed the opportunity to familiarize themselves with the apparatus, they will enter it of their own accord. The same authority also mentioned that the rat should be placed into the cage anaesthetized. Animals in such cages are able to move their limbs, change position and have an uninterrupted view. Waynforth advised that further restraint of rats is unadvisable as they may become highly stressed. In this context, Bonfils et al (1959) recorded the beginnings of gastric ulcerations in rats subject to profound restraint within 24 hours. However, ulcerations were not observed in the small intestine.

For the reasons outlined, it was decided that an apparatus similar to a Bollman cage would be suitable for the period of the drug infusion. Accordingly, appropriately sized PVC piping was sectioned and placed into the cages to allow the rats to familiarize themselves with these devices. These were readily accepted, the rats being happy to seek refuge in them when approached by laboratory staff. It was also noted that animals when placed in the tubes and restrained by taping their tails, ate and drank normally. They failed to lose any weight over a 48 hour period, produced solid stools and appeared in no distress, as evidenced by the absence of energetic efforts to escape their confinement. On the basis of the preliminary trials and subsequent experiments, it was felt that confining animals in such an apparatus for the period of the infusions did not place undue stress on the animals. Although not the ideal method of restraint, this method was deemed a

humane and satisfactory way of preventing interference with an infusion catheter for a short period.

Toxicity Studies

A was discussed in the literature review, the toxicity of methotrexate in rats appears to be variable and dependent on several factors. These include the particular strain of animal used, their size, sex and age. The route of drug delivery and dose regime also have a bearing on the degree of toxicity seen. Because of these factors it was necessary to conduct a series of trials in order to determine the toxicity of methotrexate, when delivered by an intraperitoneal infusion method to these animals.

During the course of this experiment no animal died prematurely from effects other than those ascribable to the methotrexate. All animals appeared to tolerate the anaesthetic and period of restraint well. The control animals lost, on average, only 2.8% of their body weight while in the infusion apparatus. Each animal began to gain weight on return to its cage and each had exceeded its preoperative weight after the passage of 7 days. These animals showed no noticeable signs of stress and suffered no ill effects from their experience.

By way of contrast, reviewing weight data from all of the animals which received a dose of methotrexate revealed an average weight loss of 4.2% during the infusion period. It could be speculated that this difference was caused by early effects of the drug, perhaps causing anorexia, increased gut motility or increased urine output. The 10 animals which were entered into the leucovorin rescue group lost on

average 6.4% of body weight during their period of restraint. It is not clear whether this difference relates to reduced food intake during a longer period of restraint or, as would seem more likely, the beginnings of the toxic effects of the methotrexate.

Following methotrexate infusion, a number of toxic effects were observed in the animals. These were anorexia, an untideness of the coat, pigmentation about the eyes, snout and feet, diarrhoea, lethargy and weakness. With fatal doses of methotrexate these were followed by dehydration, emaceation, if the animal survived for a number of days, and death. The general course of intoxication observed in this study is in agreement with the descriptions provided by previous investigators, notably Ferguson et al (1950), Vitale et al (1954), Loehry and Creamer (1969) and Robinson et al (1966) all of whom used methotrexate regimes different from an intraperitoneal infusion (Table 1.2). No additional features not mentioned by these authorities, were noted.

Alopecia has not been reported to be a feature of methotrexate toxicity in rodents. Animals in this experiment did not lose hair, but ruffled coats affected all of the morbidly toxic animals. This feature was perhaps caused by the cessation of the normal preening activities of the animals as they became unwell.

Animal subjected to the universally lethal doses of 100, 50 and 37.5 mg/kg of methotrexate all suffered from the full spectrum of toxic effects and died, or were sacrificed, by the sixth day after infusion (with the exception of one animal which survived for 9 days). All of these animals survived for at least two days after the infusion period. This result is consistent with the findings of Philips and Thiersch

(1949) who also noted that the course of intoxication was not changed by exceeding the LD 50 dose by one order of magnitude.

Animals suffered from progressively less severe toxic effects as the dose of drug was reduced. Accompanying this change was more variability in the toxic effects observed between animals which had received a similar dose of methotrexate. This was also reflected in the fact that some animals died and others survived doses ranging from 10 mg/kg to 30 mg/kg. Toxic effects were least severe in the 5 mg/kg group. No animal succumbed to this dose and the most severe toxic effect, diarrhoea, occurred only transiently in some of the animals.

While all animals in the 10 mg/kg and 25 mg/kg groups suffered from toxic effects, a number of these animals were able to recover. Those animals which succumbed generally survived longer than those in the higher dosage groups. The average time of survival of those animals which died in the 10 mg/kg and 25 mg/kg groups was 7.5 days. Those animals which received 50 mg/kg or 100 mg/kg survived, on average, only 4 days. This finding is in agreement with the study of Millington et al (1962a) where rats receiving a lethal dose of aminopterin also died on or about the fourth day. In the present study animals alive on the eighth day survived on all but 3 occasions. Only two animals died after the tenth day. Most animals dying from methotrexate toxicity succumb to gastrointestinal damage (Robinson et al 1966). Animals dying after 10 days, when diarrhoea had ceased, may have died from the failure of another system, such as the bone marrow, or from secondary infection.

In this experiment the clinical toxicity of methotrexate appeared to rise sharply over a relatively narrow range of dose from the LD 10 at

10 mg/kg to the LD 90 at 30 mg/kg. The accuracy of this observation could be questioned on a statistical basis, owing to the small number of animals in the study. However, it was felt unnecessary to enter more animals into toxicity trials because of constraints on animal usage and when, for the purpose of the experiment, approximate values would suffice.

The LD 50 determined in this experiment was calculated to be approximately 25 mg/kg. This is 0.25 of the dose of methotrexate when administrated as a single dose mentioned by Rall (1963). It is 2.5 times greater than the LD 50 determined by Sindram et al (1974), when they continued their infusion for 7 days. The finding that the 24 hour infusion has a toxicity between that of a single dose and that of a one week infusion, is consistant with expectations. The maintenance of high serum levels for long periods above the threshold for DNA synthesis inhibition in the small intestine, predisposes to greater toxicity.

Serum assay of methotrexate was undertaken in order to define the schedule of the drug best able to rescue the animals. The results of this assay were variable. The maximum level of drug detected occurred immediately at the end of the infusion. This level of 7.4×10^{-6} M would enable rescue to be attempted at equimolar doses of leucovorin. One specimen from this time period had a level an order of magnitude less. Methotrexate was still present at levels which would be expected to inhibit rodent intestinal DNA synthesis 24 hours later, but had fallen to indectable levels after a further 24 hours. The variable results obtained in the serum assay in this study may have reflected real variations in the levels of methotrexate attained in the experiment. Alternatively, errors in the collection of the samples, or in their

handling, may explain this finding. For example, methotrexate in serum is light sensitive and some light contamination may have occurred. Additionally, haemolysis can be a problem with cardiac puncture and it is possible that haemolyzed cells in the samples interfered with the accuracy of the assay process.

The indwelling catheter was a convenient way of delivering leucovorin intraperitoneally without further trauma to the animals. The dose of leucovorin given over 24 hours in the rescue phase of this study was equal to the methotrexate dose. This could be considered a low dose of leucovorin, but it was important to use as low a dose as possible, as high doses have a greater potential to rescue neoplastic cells, as well as the normal cells.

In terms of survival, some success was obtained in attempting to rescue the rats from the LD 90 dose of methotrexate. Whereas only one rat survived this dose in the unrescued group, 8 survived when given leucovorin. In no case was an animal rescued entirely from the toxic effects of methotrexate. All animals experienced a period of diarrhoea and weight loss. The leucovorin group suffered a higher initial weight loss than the nonrescued group, possibly because they were subjected to experimental conditions for 24 hours longer. The maximum weight loss of the surviving rescued animals ranged from 9.8-19.5% of initial body weight. This is very similar to that experienced by surviving animals in the 10 mg/kg methotrexate group. The rate of weight loss is comparable between the rescued and unrescued 30 mg/kg groups of animals (Figs. 4.10, 4.11), but a larger number of animals were able to recover from the period of weight loss if they had received the rescue agent. On raw survival data, the regime with leucovorin rescue would appear to be

significantly less toxic and achieved the aim of increasing the survival of rats receiving the LD 90 dose. Only a mild degree of reduction in the clinical toxicity was achieved when weight changes and toxic signs in the rats were considered.

Small Intestinal Studies

A number of difficulties were experienced with the preparation and examination of the intestinal tissue. It was found that artefacts caused during sectioning were a problem in many tissue sections. Some of these had to be recut with great care in order to minimize artefact. Tips of intestinal villi were particularly prone to damage during sectioning. This posed some difficulties as such artefacts could be misinterpreted as experimentally induced changes.

An attempt was made to quantify the number of goblet cells on the villi. Variations in the staining of mucin made the cells more obvious in certain sections, and more difficult to identify in others. Additionally, where the villus height became depressed, the area counted included the intervillous area which often contained a disproportionately high goblet cell population, when compared with the villus tips. The results of this attempt were not considered to be accurate, so no conclusions were reached about the behaviour of these cells under the effects of methotrexate.

Sections from the terminal part of the jejunum were not included in the quantative examination of the tissues. This was because there was considerable variation between the histology of tissue from this area and from the tissue adjacent to the duodenum. The further one travels

along the small intestine the smaller are the villi. Altmann (1972) recorded nearly a three-fold decrease in villus size between the duodenum and the ileum. Altmann and Enesco (1967) found that the life span of epithelial cells is longer in the duodenum compared with the ileum, by a factor of two to one. Because of these differences there is a difference in the response of the tissues to methotrexate. Epithelial denudation is likely to start earlier in the distal portion of the small intestine. This trend was observed in the tissues examined during the present experiments. It was also noted by Vitale et al (1954) and Altmann (1974). Recovery from the effects of the drug proceeded correspondingly slowly in the more distal tissue.

It was felt that for the purposes of this experiment it was more important to study the response of a relatively uniform piece of tissue to the drug, rather than to compare the responses of different tissues. Therefore, the distal specimens were not studied in detail.

A number of factors have been reported to cause histopathological changes in the gastrointestinal tract of rats (Greaves and Facconi 1984). Those which may be of relevance to this study are starvation, as demonstrated by Altmann (1972), stress (Bonfils et al 1959) and the cytotoxic drugs. Altmann (1972) found that starvation reduced villus size, crypt size and mitotic pool in the small intestine of rats within 3 days. In the present study food and water were made available during the infusion period and immediately afterwards, in order to minimise any intestinal changes due to starvation. Where animals received methotrexate and subsequently became anorexic, the effects of the reduced food intake could not be distinguished from the direct effects of the methotrexate. Therefore, the possible effects of starvation on

small intestinal morphology could not be entirely discounted as a variable in this study. However, judging by the results of Millington et al (1962a) variations in food intake may not have greatly changed the histological findings in the present study. They included a starved control group of rats in their study of the effects of methotrexate on small intestine and recorded no histological changes in villus size or epithelial cells between these animals and a fed group of control animals.

In the present study animals were included which received only normal saline infusion. These had been subjected to the stress of the experimental procedure for 24 and 48 hours respectively. No microscopic changes from normal were observed in the jejunum of these animals. Examination of the stomach was not made. The anaesthesia and period of restraint used in these experiments was concluded to be relatively benign, on the basis of both gross and microscopic findings.

Group 1, 10 mg/kg Methotrexate

The changes observed in the rat jejunum over 4 days following infusion of 10 mg/kg of methotrexate consisted of a period of mucosal degenerative changes lasting at least up to 48 hours, followed by a period of recovery towards a normal histological picture at 96 hours. The tissues of the submucosa and muscular layers were not obviously involved in these processes. Of greatest significance were the changes observed in the crypts of Lieberkuhn. Cell death was a prominent feature in the crypts until 48 hours post infusion (Figs. 4.25, 4.26). Cellular death was not noted in the lamina propria or epithelium although some abnormal degenerative changes in villus tip cells were seen (Fig. 4.31)

prior to their exfoliation. This finding is consistent with the known action of methotrexate against cells synthesizing DNA. It is expected to spare differentiated non-dividing cells.

Mitoses were not seen in the crypts of Lieberkuhn until 48 hours after the methotrexate infusion had ceased and then, only in one animal at that time. At 96 hours they were observed in every section examined and were scored as multiple in number. Mitoses were not seen in the earliest examined sections (0 hours). This was 24 hours after the beginning of the methotrexate infusion, at a time when a steady level of serum methotrexate might have been attained. It can thus be inferred that this level was sufficient to cause a cessation in mitoses in rat jejunum. A loading dose of 10% of the total dose had been given at the commencement of the infusion period and this may have been sufficient to terminate DNA synthesis soon after its injection. Margolis et al (1971) observed a reduction in DNA synthesis of 99% just one hour after injection of methotrexate into mice. Pinedo et al (1977) stated that a priming dose of 5% mg/kg of methotrexate administered intraperitoneally was sufficient to induce an immediate inhibition of dihydrofolate reductase activity and that this inhibition lasted the 6-8 hours required for a methotrexate infusion to produce a plateau plasma level.

Vitale et al (1954) noted a reduction in mitoses 4.5 hours after subcutaneous injection of aminopterin (240 ug) and the absence of mitotic activity after 6 hours. Loehry and Creamer (1969), Margolis et al (1971) and Altmann (1974) all noted a marked reduction in crypt cell mitoses within 24 hours of administering methotrexate. Loehry and Creamer (1969) measured the number of mitoses in crypts of rats injected intramuscularly with 7.5 mg/kg of methotrexate and recorded minimal

mitotic activity for two days after this dose, then an increase on the third day to near baseline levels. On days 4 and 5 mitoses were up to 5 times more numerous than normal. By 7 days levels had returned to within the normal range. Although no attempt was made during the present study to accurately quantify the number of mitoses it is felt that the findings are compatible with those of previous studies. Nevertheless, future quantitative studies are needed to confirm this view.

The fate of the crypts of Lieberkuhn following 10 mg/kg of methotrexate was variable. The change in crypt shape observed is secondary to the migration of cells out of the crypt onto the wall of the villus, where they assume their roles as either goblet cells or absorbtive cells. The lost crypt cells are not replaced as mitotic activity ceases, but the process can continue for some time owing to the large cell population of the crypts. In this study, crypt cell numbers appeared to decrease as did the size and number of the crypts (Fig. 4.25). Fewer crypts could be seen communicating with the lumen. A possible explanation for these observations is that despite the decrease in crypt cell numbers, the basal cells did not move towards the gut lumen. They were thus left as small populations of crypt cells in the lamina propria. As crypt cells died from the effects of methotrexate, the number of cells in the crypts would have been further reduced. Progressive death of crypt cells led to cystic degeneration of the crypts. These microcysts contained some cellular debris and some intact cellular structures (Fig. 4.41). These were interpreted as being dying crypt cells, but authors such as Vitale et al (1954) and Wynn Williams (1961) have mentioned the presence of polymorphs in the cystic crypts. These structures were eventually surrounded by a single layer of flattened cells (Fig. 4.48). In this form they were difficult to

distinguish from lymphatic vessels. In the worst affected of the Group 1 animals (at 48 hours) it appeared that cystic crypts had become continuous with lymphatic vessels (Fig. 4.47). Whether this in fact occurred could not be confirmed at the optical microscopic level.

Changes in the lamina propria consisted of oedema and lymphatic dilation and some changes in the inflammatory cell population. Inflammatory cells are prominent in normal jejunum. In some of the sections examined, particularly from the animals sacrificed after 24 hours, there was an apparent, but subjectiveley assessed, increase in these cell numbers (Fig. 4.31). This was not a universal finding at this or any other time interval. Lymphatic and lacteal dilation was in evidence in some specimens as early as 0 to 12 hours and in 24 and 48 hour specimens, was a constant feature. Much of the lamina propria was occupied by lymphatic structures in the worst affected examples (Fig. 4.35). Oedema was most noticeable under the tips of the villi but again, was not a constant finding and was variable in extent when present.

Changes in the morphology of the villi and of the epithelium are thought to occur, not as a direct result of the methotrexate affecting these structures, but rather, from the effects of the drug on the crypts of Lieberkuhn. Epithelial cells lost from the tips of the villi during the normal process of exfoliation, continue to be replaced for some time by those cells migrating out of the crypts. Differentiation into absorbtive cells also continues, so villus epithelium is maintained in form for some time. Altmann (1974) confirmed the details of this phenomenon by studying the progress of radiolabelled cells out of the crypts, despite cessation of DNA synthesis. Later, terminal cell loss continues but no replacement cells are available. At this stage changes

in the shape of the epithelial cells are noticed. In the present investigation this occurred 48 hours after the cessation of DNA synthesis. These cells became less regular in shape and were later replaced by cuboidal shaped cells. The latter probably represented epithelial cells which failed to differentiate into functioning absorbtive cells. Goblet cells became uncommon on the villus tips of those animals sacrificed after 48 hours. As the number of cells available to cover the villi was reduced, so too did the size of each villus decrease, reducing the potential intestinal absorbtive area.

Recovery from the severe methotrexate induced changes seen at 48 hours was well advanced in the sections examined from the 96 hour animals. An apparent new epithelial cell population was observed, replacing the atrophic cells remaining from the period of mitotic inhibition. The rapid recovery of small intestinal epithelium after mitotic activity had been re-established, has been demonstrated by Taminiau et al (1980) who measured an increase in mitotic rate in the crypts of Lieberkuhn of rats (at 96 hours) recovering from methotrexate toxicity. These investigators recorded an increased rate of migration of cells out of the crypts and along the villi. Recovery of a normal epithelial cell population is very rapid as long as a significant population of crypt cells survive the period of mitotic inhibition. It is anticipated that had a group of rats been sacrificed one week after the methotrexate infusion, a complete or near complete recovery to normal form would have been observed in the intestinal mucosa.

Group 2A, 30 mg/kg Methotrexate

Four animals from this group failed to survive until their scheduled time of sacrifice at 96 hours and one week. This was not

surprising as only half of the rats receiving 30 mg/kg of methotrexate in the toxicity trial phase of the experiment survived for the lesser time and only one survived for over a week after infusion.

A similar pattern of mucosal degeneration was observed in this group as was seen in the preceeding animals which had received the lower dose of drug. However, the changes were more rapid, more severe and no recovery from them was observed. Severe degeneration of many crypts of Lieberkuhn was noted in the earliest group examined. This had occurred in the 48 hours since the presumed interuption of DNA synthesis. At this time villus height was already noticeably reduced and epithelial cells were of a cuboidal shape. After a further 24 hours crypt morphology was severley disrupted and the epithelium, which was now composed of a single layer of flattened cells, was fragmenting. Forty eight hours after the infusion period no villi could be identified. The epithelium was incomplete and no signs of recovery were evident. Because in this series of animals a course of acute toxicity was recorded, it is postulated that mitotic inhibition after methotrexate injection must have been rapid and complete and that no recovery of DNA synthesis occurred before the death of the animals.

The degenerative changes in the crypts of Lieberkuhn were severe in this group of animals, but the epithelium of the mucosal lining would appear to be the site where the major changes leading to the death of the animals occurred. The change in morphology observed in the lining epithelial cells is an interesting phenomenon. The cells assumed a cuboidal form and later, as their numbers decreased further, became flattened into a squamous configuration. Millington et al (1962a) suggested that the remaining epithelial cells deform themselves in order

to preserve the integrity of the epithelial covering of the mucosa. Altmann (1972) stated that migration of the epithelial cells eventually fails to continue. He felt that the eventual loss of epithelial cells is caused by death of the epithelial cells rather than their forced exfoliation as would occur on normal villi. Death is due to the natural termination of a short life span, (40-70 hours, Altmann and Enesco 1967).

The loss of epithelial continuity would appear to be a significant factor in determining the fate of an animal having received methotrexate. Decreased functional capacity of the intestine can be tolerated for some days, but the loss of epithelium allows rapid electrolyte loss, dehydration and bacterial invasion (Millington et al 1962a) which have fatal consequences.

Group 2B, 30 mg/kg Methotrexate, Leucovorin Rescue

Considerable degenerative changes were observed in the tissues taken from the animals which received leucovorin in addition to methotrexate. The histological picture in some ways paralleled that of the Group 1 animals which had received the lower dose of methotrexate. The most severe changes were seen 24 hours after the end of the infusion period, that is, 48 hours after the methotrexate infusion had ceased. This was also the time when the most severe mucosal disruption was observed in Group 1. In most of the 96 hour specimens (Fig. 4.60) a considerable recovery towards a more normal histological appearance was observed and this trend continued in the one week sections where little evidence of abnormality remained. The time of maximal histological change coincided with the time of maximal weight loss, anorexia and

diarrhoea seen in the toxicity phase of the study. One animal in this group failed to survive until sacrifice at one week. Animals sacrificed at 96 hours still suffered some malaise but had resumed eating. This time coincided with the nadir in body weights seen in the toxicity study animals.

In comparison with the specimens from Group 2A, the Group 2B tissue exhibited later and less severe degenerative changes, without the appearance of ulceration. Only minor changes were seen in the 0 hour specimens, but a considerable transformation was evident in the gut of those animals sacrificed 24 hours later. Most notably, villi were markedly flattened and epithelial cells were generally of a cuboidal shape. A number of crypts had undergone cystic degeneration and lymphatic vessels were markedly dilated. Because such a gross change was observed over just 24 hours, it is considered likely that the 0 hour group was represented by 3 animals which tolerated the methotrexate well and the 24 hour group by 3 which tolerated it relatively less well.

In this regard the experiments suffered from having only small numbers entered into each group. A clearer picture of the subtleties of the histological changes, possibly could be gained by entering a larger number of animals into the experiments and sacrificing them at more frequent time intervals.

The morphology observed at 96 hours showed some variation between animals, but each specimen displayed histology consistent with the mucosa having recovered for 2-3 days from the state observed in the 24 hour group. A similar recovery was observed in Group 1 when small intestinal mucosa appeared to have been largely reconstituted during the

48-96 hour interval. The ability to monitor this recovery in the rescued animals suffered by the omission of a 48 hour series of animals.

Perhaps the observation of greatest significance in this phase of the study was the presence of a number of mitotic figures throughout Group 2B sections. While mitoses were not observed in Groups 1 and 2A at 0 and 24 hours, a small population was noted at both of these times in the Group 2B animals. By 96 hours a large population of mitotic figures had become apparent in the crypts of Lieberkuhn (Fig 4.61). From these observations, one could conclude that a population of crypt cells survived the dose of 30 mg/kg of methotrexate followed by leucovorin rescue and that these formed the basis for the rapid recovery of the mucosa when the effects of the methotrexate had subsided. A proportion of the cells must have been successfully rescued, as mitoses were seen at each time interval. This would be consistent with the findings of Sirotnak et al (1977) who noted DNA synthesis at levels of 3-5% of controls, 4-6 hours after giving a small dose of leucovorin to mice having been injected with a large dose (400 mg/kg) of methotrexate two hours previously. A major cycle of DNA synthesis was recorded in this system approximately 24 hours later. It is unclear whether the mitotically active cells observed at 24 hours in the present experiment were a result of a fall in the methotrexate levels below the threshold for dihydrofolate reductase inhibition, or whether the rescue agent was still functioning (or both). It can be postulated that Group 2B animals survived because leucovorin rescue provided a continuity of DNA synthesis of sufficient level to ensure that epithelial denudation did not occur, as was seen in Group 2A. In contrast, in Group 1 the smaller dose of methotrexate given, may have resulted in a profound reduction in DNA synthesis capacity in the small intestine for a short enough period

to allow the spontaneous recovery of the tissue, when the serum methotrexate level dropped below the threshold level.

The rescue agent appears to have been effective in preventing death of some of the animals which received the higher dose of methotrexate. Further reduction in toxic effects may have followed an increase in the dose of leucovorin or the continuation of its administration for a further period. For example, Sirotnak et al (1977) found that toxicity could be prevented in mice by administering an equal dose of leucovorin two hours after methotrexate injection. Survival of the animals receiving the same dose of methotrexate could be achieved by giving just 1/16th the dose of leucovorin. In their experiments the higher dose of leucovorin significantly reduced the ability of methotrexate to kill L1210 cells. This has implications for the present study, as if induced carcinomas are to be successfully treated, the smallest dose of rescue agent should be used which is compatible with survival of the animals.

CHAPTER 6

SUMMARY AND CONCLUSIONS

During the course of these experiments, a relatively simple method was developed and used to deliver methotrexate by infusion to laboratory rats. This method was seen to be well tolerated by the animals and thought to be a humane and practical method of conducting short term infusion experiments in these animals.

The results of this study demonstrated that:

1) The LD 50 and LD 90 of methotrexate when delivered by an intraperitoneal infusion over 24 hours to male albino rats are approximately 10 mg/kg and 30 mg/kg respectively.
2) Most rats can be rescued from the LD 90 dose of methotrexate by delivering an equimolar dose of leucovorin over the subsequent 24 hours. The rescue agent reduced the toxic effects of the drug but did not protect the animals entirely from them.
3) The two regimes of 10 mg/kg (LD 10) of methotrexate infused over 24 hours and 30 mg/kg (LD 90) of methotrexate followed by leucovorin rescue, were seen to cause a period of mucosal degeneration in the small intestine of the rats from which most were able to recover after several days.
4) When 30 mg/kg (LD 90) of methotrexate was delivered without subsequent rescue agent, a rapid progressive degeneration of the small intestinal mucosa occurred with a fatal consequence for most animals.

5) Changes in the small intestinal histology after methotrexate infusion were felt to be consequential to the inhibitory effects of the drug on DNA synthesis in the cells of the crypts of Lieberkuhn.

On the basis of these findings it is felt that the infusion model developed would prove a suitable model for studying the effects of intraperitoneal infusions of methotrexate, with or without, leucovorin in oral cancer bearing rats.

While interesting microscopic findings were noted in the various experimental groups studied, further investigation aimed at quantifying features such as changes in the mitotic rate of intestinal crypt cells and studying the ultrastructural features of mucosal epithelial cells subsequent to the methotrexate regime employed, would be desirable in future studies.

APPENDIX I

Methotrexate can be administered in various dose regimes as detailed by Bertino (1981). They can be subdivided into low (or conventional) dose, moderate dose, or high-dose.

DOSE	ROUTE	FREQUENCY	LEUCOVORIN
I. Conventional dose			
(a) 15-20 mg/m^2	po or iv	twice weekly	-
(b) 30-50 mg/m^2	po or iv	weekly	-
(c) 15 mg/m^2 x 5 days	iv or im	every 2-3 weeks	-
II. Moderate dose			
(a) 50-150 mg/m^2	iv push	every 2-3 weeks	-
(b) 240 mg/m^2	iv infusion (24 hours)	every 4-7 weeks	+
(c) 0.5-1.0 g/m^2	iv infusion (36-42 hours)	every 2-3 weeks	+
III. High-dose			
(a) 1-12 g/m^2	iv (1-6 hours)	every 1-3 weeks	+

APPENDIX II

Responses to chemotheraphy

Tumour responses are assessed by measurement of lesions prior to and after cytotoxic drug administration. Assessment can be made manually or, if the lesion is deeply situated, by CT scanning or ultrasound.

Responses are categorized by convention into four grades;

(1) CR ; complete response refers to the clinical disappearance of measurable disease.

(2) PR ; partial response refers to a reduction in the size of the measured malignant lesion of 50% or more, without the development of new lesions.

(3) NC ; no change denotes less than a 50% reduction in the size of the malignant lesion.

(4) PD ; progressive disease describes an increase of greater than 25% in the measurable disease or the appearance of a new lesion during the therapy.

APPENDIX III

Clinical staging of carcinoma of the oral cavity. From the American Joint Committee for Cancer Staging and End Results Reporting (AJCCS).

Stage I:	T1	N0	M0			
Stage II:	T2	N0	M0			
Stage III:	T3	N0	M0			
	T1	N1	M0			
	T2	N1	M0			
	T3	N1	M0			
Stage IV:	T1	N2	M0	T1	N3	M0
	T2	N2	M0	T2	N3	M0
	T3	N2	M0	T3	N3	M0
	Or any T or N category with M1.					

Definition of the TNM Categories of Malignant Tumours about the Oral Cavity

T - Primary Tumour
- TIS - Carcinoma in situ.
- T1 - Tumour 2 cm. or less in greatest diameter.
- T2 - Tumour greater than 2 cm. but not greater than 4 cm. in greatest diameter.
- T3 - Tumour greater than 4 cm. in greatest diameter.

N - Regional Lymph Nodes.
- N0 - No clinically palpable cervical lymph node(s); or palpable node(s) but metastasis not suspected.
- N1 - Clinically palpable homolateral cervical lymph node(s) that are not fixed; metastasis suspected.
- N2 - Clinically palpable contralateral or bilateral cervical lymph node(s) that are not fixed; metastasis suspected.
- N3 - Clinically palpable lymph node(s) that are fixed; metastasis suspected.

M - Distant Metastasis
- M0 - No distant metastasis.
- M1 - Clinical and/or radiographic evidence of metastasis other than to cervical lymph nodes.

APPENDIX IV

Preoperative chemotheraphy regime used to treat patients with squamous cell carcinoma of the oral cavity. Based on recommendations of Capizzi et al (1970).

1. 24 hours infusion:

   1000 ml. 5% glucose with 240 mg/m^2 MTX.

2. directly after 1, 12 hours infusion:

   500 ml 5% glucose with 75 mg citrovorum factor.

3. then:

   15 mg citrovorum factor orally every 6 hours (in total 6 x 15 mg).

   Na HCO_3 4 x per day 1 gram orally, start 1 day before MTX infusion, till 2 days after MTX infusion.

   repeat: every week for three weeks.

APPENDIX V

Tumour induction with 4NQO.

Whereas many implantable tumours are available for study in rodents, no model is freely available for the implantation of oral squamous cell carcinoma. The oral cavity has also proven to be a difficult site for the experimental induction of squamous cell carcinoma (LEVY 1963). In the 1950's it was shown to be possible to induce tumours in hamster cheek pouches using the soluble carcinogen 7-12-dimethyl-benz(a)anthracene (DMBA) (SALLEY 1954). Chino, Fugino and Imai (1963), succeeded in the production of oral carcinoma in mice by the application of 4-nitroquinolene-N-oxide (4NQO). Wallenius (1965) produced carcinoma in the oral cavity of Sprague-Dawley rats after painting on DMBA and later, Wallenius and Leckholm (1973) used 4NQO to produce carcinomas in the same animals. This appeared to be a reliable if tedious method, requiring as much as six months of carcinogen application to produce the tumour.

In 1981 Wong confirmed the reliability of this method using Porton strain albino rats. The features and histopathology of these lesions as described by Wallenius and Leckholm (1973) and Wong (1981) are worthy of reiteration.

The initial reaction seen in the fifth month was reddening of the palate, followed by increased keratinization. By the sixth month ulcerations were present. The ulcerations expanded over the succeeding months. Histopathological examination revealed highly differentiated squamous cell carcinoma. The tumour infiltrated into the jaw bones of

the animals. The central parts of the lesion showed keratinization while the periferal regions had more anaplastic features.

Wong (1981) succeeded in producing lesions on the palate and the tongue by applying the carcinogen to the palate only. He recognised three types of malignant lesions. Firstly a Verrucous carcinoma in the vault of the palate. Secondly an invasive squamous cell carcinoma of the alveolar process and thirdly, infiltrating carcinoma of the posterior tongue was present in some animals. The carcinomas were described as well differentiated.

Wallenius', animals exhibited weight loss and loss of activity from the sixth experimental month. Anorexia ensued, and later breathing difficulties. This was ascribed to the increase in size of the tongue.

Details of factors which may have a bearing on the succeptibility of this tumour to methotrexate such as mitotic activity and the permeability of the tumour cells to the drug are not available. No studies of cell kinetics were made. In the hamster cheek pouch carcinoma, cells have a shorter cell cycle and the stages of the cell cycle are each shortened (Nagamine 1978). Toto and Swaske (1973) mentioned that DNA synthesis activity in the basal cell layer is markedly increased from normal.

APPENDIX VI

Manufacturer's specifications of diet and cage

DIET (PELLET)

Charlicks Feeds

Mouse Ration

Min. Crude Protein.....21.0%

Min. Crude Fat.....3.0%

Max. Crude Fibre.....7.0%

Min. Vitamin A.....100000 I.U./kg.

Min. Vitamin D.....2500 I.U./kg.

Vit. E.....20 I.U./kg.

Vit. K.....1.1 mg/kg.

Vit. B_{12}.....10 mcg/kg.

Max. Added Salt.....1.0%

Ingredients:

Wheat, Barley, Lupins, Peas, Wheat Offal Lucerne, Meat Meal, Soyabean, Sunflower Meal, Yeast, Cotonseed, Meal, Peanut Meal Fish Meal, Salt, Calcium, Phosphate, Vitamins, Minerals, Tritiale, Oats, Groats, Sorghum

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Manufactured by:

Milling Industries Ltd. South Australia.

CAGE

NORTH KENT PLASTIC CAGES LTD. ENGLAND.

RB3 cage
Polypropylene
Stainless steel top
Size: 45 cm x 28 cm x 22 cm

APPENDIX VII

Histological Techniques

Tissue Fixation

For the purpose of this investigtion, specimens were fixed in neutral buffered formal saline (n.b.f.s.). The constituents of the fixative are:

Formaldehyde solution	100 mls
Sodium dihydrogen phosphate	3.5 gms
Di-sodium hydrogen phosphate	6.5 gms
Distilled water	900 mls

Embedding

The specimens were passed through the following reagents:

at 37°	1.	Alcohol	70%	1 hour
	2.	Alcohol	80%	1 hour
	3.	Alcohol	90%	1 hour
	4.	Alcohol	Absolute	1 hour
	5.	Alcohol	Absolute	1 hour
	6.	Alcohol	Absolute	1 hour
	7.	Chloroform		1 hour
	8.	Chloroform		1 hour
	9.	Wax		2 hours
	10.	Wax		2 hours

APPENDIX VIII

Histological Techniques

(A) Haematoxylin and Eosin Staining.

Remove Wax.

(i)	xylol	2 minutes
(ii)	xylol	2 minutes
(iii)	absolute alcohol	2 minutes
(iv)	absolute alcohol	2 minutes
(v)	dip sections in tap water	

Staining.

(vi)	haematoxylin	10 minutes
(vii)	running tap water	5-10 minutes
(viii)	dip in 1% HCl in 70% alcohol	
(ix)	running tap water	5-10 minutes
(x)	eosin	45 seconds

Dehydration.

(xi)	absolute alcohol	1-2 minutes
(xii)	absolute alcohol	1-2 minutes
(xiii)	xylol	1-2 minutes
(xiv)	xylol	1-2 minutes
(xv)	mount with Depex	

(B) PAS stain with prior diastase digestion.

Diastage Digestion.

(i) Sections to water

(ii) Place slides in preheated diastase solution for 1 hour at 37 degrees centigrade

(iii) Stain with PAS or other stain

Periodic Acid Schiff (Ref. Lynch et al. (1969) Medical laboratory technology and clinical pathology pp.1088-1089).

(i) Sections to water

(ii) Oxidise in 0.5% aqueous periodic acid

(iii) Rinse in tap water, then distilled water

(iv) Place in Schiff's Reagent for 15 minutes

(v) Wash in running tap water (5-10 minutes)

(vi) Counter stain with Mayer's haematoxylin

(vii) Differentiate in 1% acid alcohol

(viii) Blue in running tap water

(ix) Dehydrate clear and mount

Appendix IX: Proforma used to record morphological changes in intestinal tissue.

SECTION	VILLI			EPITHELIUM			CRYPTS									MUCOUS CELL	LAMINA PROPRIA		
	NORMAL	ABNORMAL		NORMAL	ABNORMAL		MITOSES			CELL DEATH		FORM				NUMBER	NORMAL	ABNORMAL	
		MINOR	MAJOR		MINOR	MAJOR	ABSENT	PRESENT	MULTIPLE	PRESENT	ABSENT	NORMAL	MINOR	MAJOR				MINOR	MAJOR

Appendix X: Details of Methotrexate Assay

5cc. of blood was collected by cardiac puncture from each animal and placed in a vial containing EDTA for anticoagulation. These specimens were centrifuged and 50 ul of serum was collected for analysis. The specimens were stored in darkness at 5°C.

EMIT^{R} Methotrexate Assay is a radioimmunoassay technique which is available in kit form (SYVA^{R}). It is able to measure samples containing between 3×10^{-7} and 2.6×10^{-3} M/litre of methotrexate (GUSHAW 1978).

PRINCIPLES OF TECHNIQUE

A drug is labelled with an enzyme. When this complex is challenged by an antibody against the drug, the activity of the enzyme is reduced. The free drug in the sample competes with the enzyme labelled drug for the antibody and decreases the antibody induced inhibition of the enzyme. Enzyme activity is measured by an absorbance change which results from the enzymes catalytic action on a substrate and this correlates with the concentration of free drug.

In EMIT Methotrexate Assay serum is mixed with a reagent containing antibodies to methotrexate together with substrate for the enzyme glucose-6-phosphate dehydrogenase. Methotrexate labelled with the enzyme is then added. The labelled drug is able to combine with any unfilled binding sites and this reduces the residual enzyme activity. This activity is directly related to the concentration of methotrexate present in the serum of plasma. The enzyme converts nicotinamide adenine dinucleotide (NAD) to NADH resulting in an absorbance change which can be measured spectrophotometrically.

Appendix XI(a): Weight changes and fate of rats following a twenty four hour infusion.

Normal Saline

	DAYS																				
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Weight gms	442	430	435	437	439	441	442	445	448	449	449	452	454	455	457	459	459	461	461	462	463
	458	451	451	455	459	461	462	462	463	463	462	464	465	467	470	470	471	470	471	472	472
	449	427	431	438	446	446	448	451	451	451	452	452	453	454	454	455	456	457	459	458	460
	428	412	416	424	428	430	431	431	432	433	433	432	433	435	435	437	438	437	438	437	438
	387	379	382	385	387	390	392	392	395	398	402	404	403	404	404	406	408	409	412	412	412

Appendix XI(b): Weight changes and fate of rats following a twenty four hour infusion.

100 mg/kg methotrexate

	DAYS																			
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Weight gms	372	356	344	337	326	deceased															
	395	372	360	deceased																	
	393	378	375	375	356	deceased															
	380	362	333	308	sacrificed																
	361	342	326	299	sacrificed																

Appendix XI(c): Weight changes and fate of rats following a twenty four hour infusion.

50 mg/kg methotrexate

	DAYS																			
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Weight gms	422	409	403	394	362	sacrificed															
	445	435	416	409	deceased																
	395	378	363	deceased																	
	412	395	390	380	deceased																
	456	449	421	402	sacrificed																

Appendix XI(d): Weight changes and fate of rats following a twenty four hour infusion.

25 mg/kg methotrexate

	DAYS																				
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Weight gms	375	350	347	340	336	316	291	278	265	258	258	252	236	237	sacrificed						
	468	450	437	423	403	388	387	368	deceased												
	400	381	354	350	341	332	330	308	304	305	315	329	325	328	330	341	346	353	359	368	373
	398	373	362	349	341	336	325	deceased													
	468	455	441	429	418	406	390	396	396	402	409	414	415	418	423	432	435	438	451	458	465
	425	409	380	375	373	348	342	344	341	343	346	350	355	361	367	342	381	388	396	406	414
	422	407	403	399	395	365	373	378	385	384	384	385	389	393	404	407	412	417	419	426	430
	384	374	373	367	353	deceased															
	402	376	375	370	334	350	364	362	368	372	379	384	396	402	401	412	418	421	420	421	421
	397	369	365	365	362	362	360	361	363	370	376	379	387	392	395	398	397	400	406	407	409

Appendix XI(e): Weight changes and fate of rats following a twenty four hour infusion.

10 mg/kg methotrexate

	DAYS																				
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Weight gms	355	336	335	326	317	308	327	322	324	325	329	345	348	359	356	362	370	378	380	384	386
	405	390	386	372	366	344	342	333	deceased												
	402	383	382	374	362	354	338	339	350	deceased											
	415	399	396	392	378	371	370	359	363	376	375	376	379	381	387	391	393	395	399	399	408
	423	392	387	389	380	368	376	383	369	376	376	382	381	386	391	393	406	412	421	428	447
	405	384	383	380	376	367	366	367	373	374	379	380	383	389	389	392	396	398	402	406	408
	450	441	422	412	406	399	393	392	393	392	392	395	399	402	403	403	410	413	420	425	437
	445	430	416	410	401	391	392	390	391	389	388	386	387	388	388	388	390	393	402	410	416
	452	436	423	420	419	417	416	416	418	420	425	426	428	431	431	434	434	437	442	448	449
	402	386	368	361	353	340	341	342	340	338	340	341	342	345	346	348	351	362	369	387	392
	430	409	401	395	383	368	360	371	358	362	367	370	373	375	381	382	388	391	403	406	410
	402	380	372	367	339	334	328	329	337	339	342	350	351	357	367	371	376	380	382	386	394
	392	376	364	360	346	340	330	331	334	335	340	342	344	347	349	353	365	372	380	383	390
	350	328	317	311	303	295	291	291	294	295	304	305	312	314	318	328	329	332	342	349	354
	355	342	337	340	322	316	327	328	330	337	340	351	361	374	370	365	366	372	370	370	371

Appendix XI(f): Weight changes and fate of rats following a twenty four hour infusion.

5 mg/kg methotrexate

	DAYS																				
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Weight gms	455	435	426	421	420	421	424	425	430	432	433	437	439	442	448	451	452	456	458	460	460
	470	466	454	447	435	439	441	441	443	445	449	449	452	453	454	458	460	462	462	464	462
	478	464	457	449	444	442	444	444	448	449	452	453	458	462	467	465	465	467	466	469	474
	454	430	428	433	440	443	444	442	447	446	449	449	451	452	453	452	457	456	457	456	446
	352	333	337	342	352	358	360	364	364	367	368	368	370	373	375	377	378	380	380	384	385
	378	360	354	349	346	343	348	353	362	363	371	372	374	376	376	379	381	382	382	382	382
	406	380	371	351	356	354	368	373	376	379	383	386	389	390	393	397	398	398	400	400	403
	354	345	344	340	343	345	350	354	358	357	358	358	359	361	360	361	363	363	364	363	365
	433	417	420	423	424	424	424	426	427	426	429	428	430	431	430	431	434	432	434	436	435
	423	412	411	415	422	426	430	435	437	436	442	444	446	445	446	447	448	448	449	450	448

Appendix XI(g): Weight changes and fate of rats following a twenty four hour infusion.

37.5 mg/kg methotrexate

	DAYS																			
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Weight gms	392	372	361	344	306	sacrificed															
	418	400	394	380	351	sacrificed															
	400	380	376	362	329	305	sacrificed														
	384	367	370	362	358	350	337	325	315	313	deceased										
	374	359	356	337	312	deceased															
	401	375	377	365	deceased																
	402	377	379	365	352	sacrificed															
	406	385	369	366	360	348	deceased														
	420	410	417	410	deceased																
	410	397	386	367	353	deceased															

Appendix XI(h): Weight changes and fate of rats following a twenty four hour infusion.

30 mg/kg methotrexate

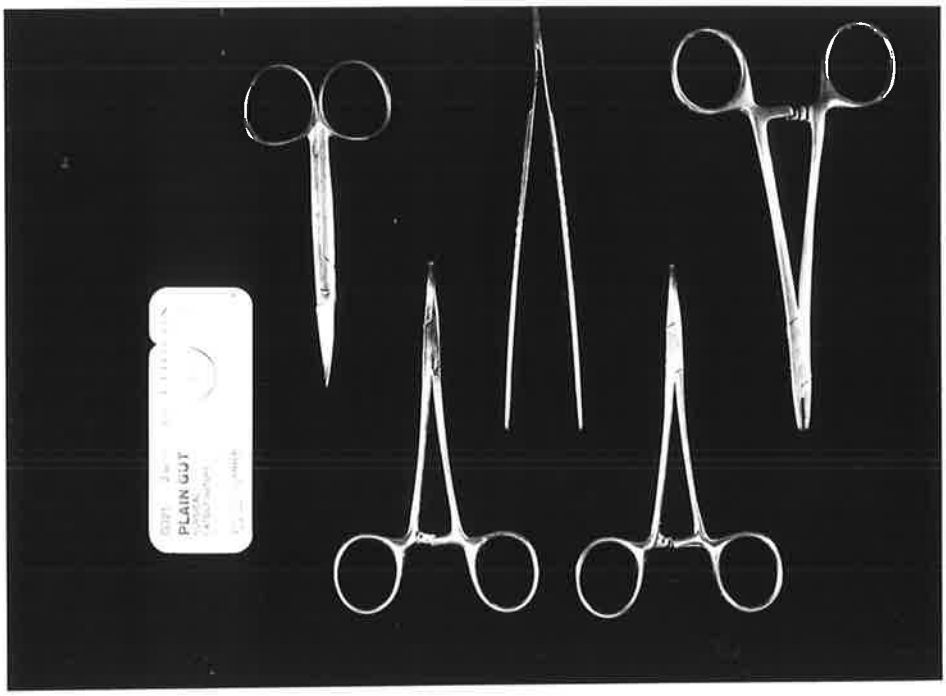
	DAYS																				
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Weight gms	382	368	363	358	deceased																
	423	412	405	387	373	361	343	sacrificed													
	389	380	375	369	358	342	330	deceased													
	447	437	432	412	deceased																
	426	412	403	391	376	deceased															
	429	418	412	403	396	390	389	386	387	389	388	395	399	403	405	405	409	413	415	415	419
	442	423	421	411	386	367	sacrificed														
	392	386	382	371	370	363	360	358	deceased												
	447	432	427	406	394	376	362	sacrificed													
	415	386	379	369	353	347	deceased														

Appendix XI(i): Weight changes and fate of rats following a forty eight hour infusion.

30 mg/kg methotrexate + leucovorin

	DAYS																				
	0	LV	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Weight gms	412		370	362	355	342	332	341	352	354	353	350	347	352	357	367	372	377	386	388	391
	450		432	417	420	417	415	403	382	deceased											
	421		390	364	348	345	341	343	349	353	354	357	360	364	367	370	376	382	389	394	406
	420		403	392	385	381	371	369	372	375	382	386	389	390	394	398	399	402	406	407	407
	451		431	405	395	392	389	379	382	387	390	396	403	407	411	418	426	429	433	440	447
	412		386	380	366	370	374	375	373	375	378	382	386	390	399	398	403	408	412	416	417
	354		334	336	326	311	306	308	307	312	314	320	323	329	339	343	351	354	357	359	360
	404		375	362	342	deceased															
	388		360	355	351	350	349	345	344	345	344	347	347	350	352	351	353	357	361	367	371
	432		398	390	385	383	384	386	386	389	390	388	391	395	396	398	399	405	417	421	425

Appendix XII: Instruments used in the surgical phases of the Experiments.



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