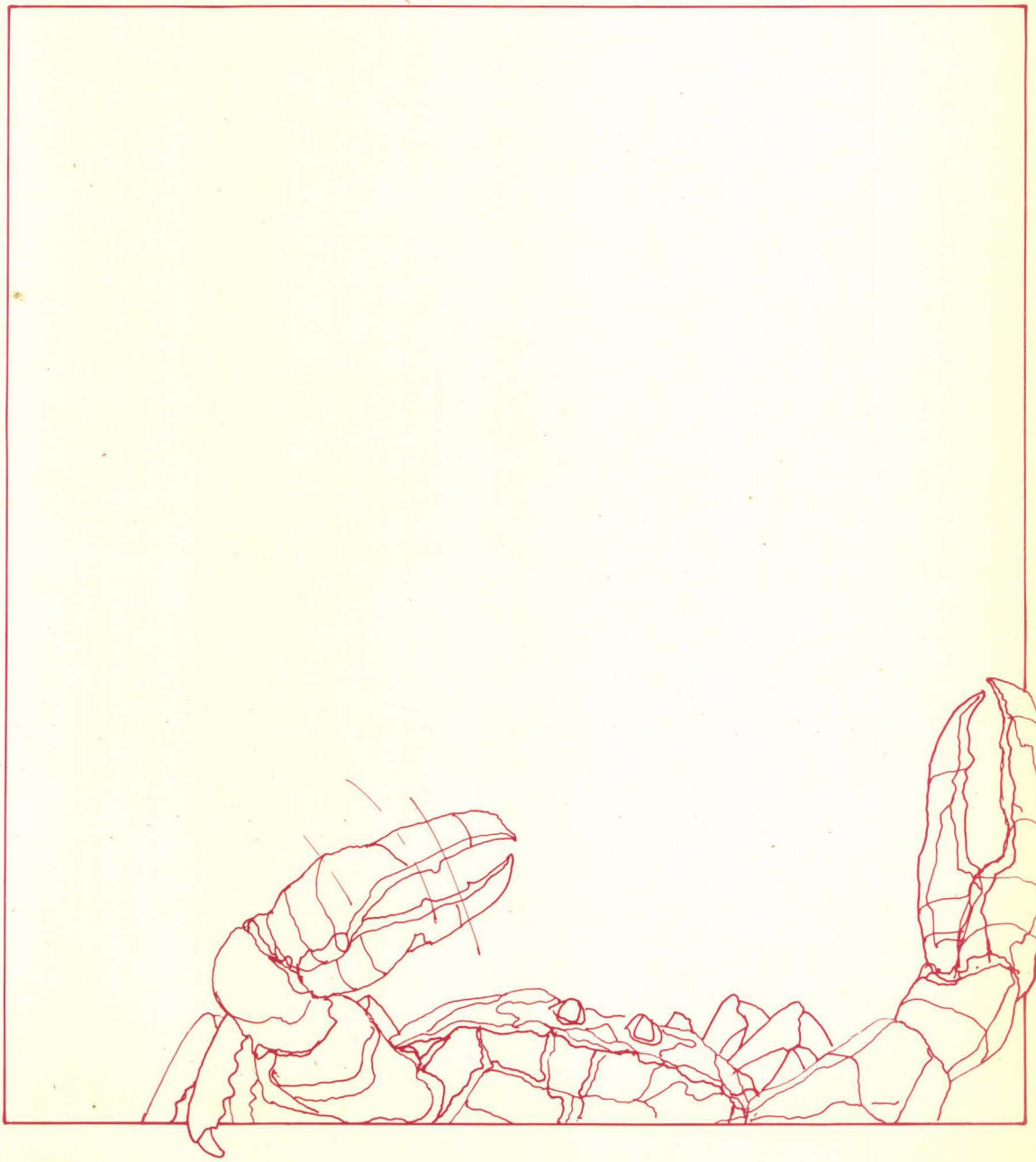
PHARMACOKINETICS OF ADRIAMYCIN IN THE RAT

P. SONNEVELD



Cover design : B.Sonneveld

PHARMACOKINETICS OF ADRIAMYCIN IN THE RAT

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR. A.A.H. KASSENAAR, HOOGLERAAR IN DE FACULTEIT DER GENEESKUNDE, VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN TE VERDEDIGEN OP DONDERDAG 26 JUNI 1980 TE KLOKKE 16.15 UUR

DOOR

PIETER SONNEVELD

Geboren te Schiedam in 1951

W.D. MEINEMA B.V. - DELFT

Promotor: Prof.Dr. D.W. van Bekkum

Referenten: Prof.Dr.Ir. O.H. Gerlach

Prof.Dr. H.M. Pinedo

STELLINGEN

1.

Voorafgaande aan de behandeling van acute myeloide leukemie kan de gevoeligheid van de tumorcellen voor Adriamycine niet alleen kwalitatief maar tevens dosimetrisch worden vastgelegd.

R.N. Buick et al.,
J. Natl. Cancer Inst. 62, 1979, 249

2.

De conclusie van Reich en medewerkers dat in oncologische patienten een gebrekkige predictie van de plasma-verdwijningscurve van Adriamycine met behulp van een drie-compartimentenmodel te wijten is aan onnauwkeurige bepalingsmethodes, is onjuist en impliceert dat een meer verfijnde modelstructuur geindiceerd is.

S.D. Reich et al.,
Cancer Chemother. Pharmac. 3, 1979, 125

3.

De cocarcinogene werking van muizeleukemievirus berust op een verhoogde lactaatdehydrogenase activiteit.

A.E. Freeman et al.,
J. Natl. Cancer Inst. 44, 1970, 65.
G.R. Andersson et al.,
Virology 99, 1979, 31.

4.

De intracellulaire concentratie van Adriamycine in het beenmerg kan direct en reproduceerbaar bepaald worden met behulp van "flow cytometry".

5.

Het gebruik van medicamenten in Nederland kan slechts op verantwoorde wijze worden teruggedrongen indien op korte termijn klinisch farmacologen worden opgeleid en ingeschakeld binnen alle sectoren van de gezondheidszorg.

6.

Gezien de schadelijke effecten van hoge doses oestrogenen, verdient postcoïtale anticonceptie door plaatsing van het"intra-uterine device" de voorkeur boven verstrekking van de zogenaamde morning-after pil.

7.

De carcinogene eigenschappen van aromatische koolwaterstoffen, zoals PolyChloroBiphenylen zou kunnen berusten op de inductie van een geconditioneerde Vitamine A deficientie, waardoor de normale "antipromoting activity" van deze stof onderdrukt wordt.

8.

Het verdient aanbeveling de huidige toelatingseisen van nieuwe medicamenten te versoepelen waar het de mutagene en carcinogene eigenschappen betreft van cytostatica die voor een phase I trial in aanmerking komen.

9.

Chronische myeloide leukemie is een lethale aandoening, waarbij momenteel een palliatieve behandeling toegepast wordt. Onderzoek naar de curatieve mogelijkheden van allogene beenmergtransplantatie in een vroeg stadium van de ziekte is gerechtvaardigd.

10.

De toepassing van de in handelskringen voorkomende principeovereenkomst "no cure, no pay" is in de geneeskunde onvoldoende onderzocht.

11.

Het ware te wensen dat CDA kamerleden beslissen of zij christenen dan wel democraten zijn.

12.

De toekomst zal uitwijzen of met behulp van kernenergie aangedreven microprocessors de beste DNA (re)combinatie van ons nageslacht zullen berekenen, of dat kinderen gewoon bij kaarslicht worden verwekt.

26 juni 1980 P. Sonneveld

PHARMACOKINETICS OF ADRIAMYCIN IN THE RAT

P. SONNEVELD

1980

PUBLICATION OF THE RADIOBIOLOGICAL INSTITUTE
OF THE ORGANIZATION FOR HEALTH RESEARCH TNO
RIJSWIJK, THE NETHERLANDS

Tegen liefde is geen kruid gewassen

Ovidius

CONTENTS

CHAPTER 1

INTRODUCTION AND RATIONALES FOR THE STUDY

The treatment of human neoplastic diseases is presently based on four major modalities: 1) radiotherapy; 2) surgery; 3) chemotherapy; 4) immunotherapy. Of these therapeutic modalities, chemotherapy is the most important and the most commonly used approach in the treatment of disseminated haematological malignancies, while the treatment of solid tumours often consists of surgery, supplemented by chemotherapy or radiation (Frei, 1978). Since the introduction of chemotherapy as a valuable adjunct in the treatment of haematological malignancies, a great variety of drugs has been used (Table 1.1). Especially the find-

Table 1.1

ANTILEUKAEMIC DRUGS IN CLINICAL PRACTICE

1946	Alkylating agents
1948	Folic acid antagonists (MTX)
1950	Steroids
1953	Antimetabolites - purine analogs (6-MP) - pyrimidine analogs (ara-C, Thioguanine)
1963	Vinca alkaloids
1967	Daunorubicin L-Asparaginase
1968	Cytosin-Arabinoside
1969	Adriamycin
1964	Combination chemotherapy

ing that combinations of several cytostatic drugs increase their therapeutic effectiveness, has led to the development and application of many different combination schedules (Fig. 1.1). It appears that the therapeutic index of cytostatic drugs alone or in combination depends on several factors, the most important being:

- sensitivity of the tumour;
- drug interaction with normal tissues;
- duration of drug effect;
- interactions between two or more drugs.

Most of the established therapeutic regimens are based on empirical observations which consider only tumour sensitivity and toxicity. However, these two phenomena are the result of many variable drug mechanisms including pharmacology and pharmacokinetics and their significance for cellular properties such as proliferative state and repair mechanisms. This is illustrated by the strong schedule dependency of cell-cycle specific drugs in the treatment of several rapidly proliferating neoplasms. In the case of acute myeloid leukaemia, a great deal of effort has been put into studying animal tumours in order to

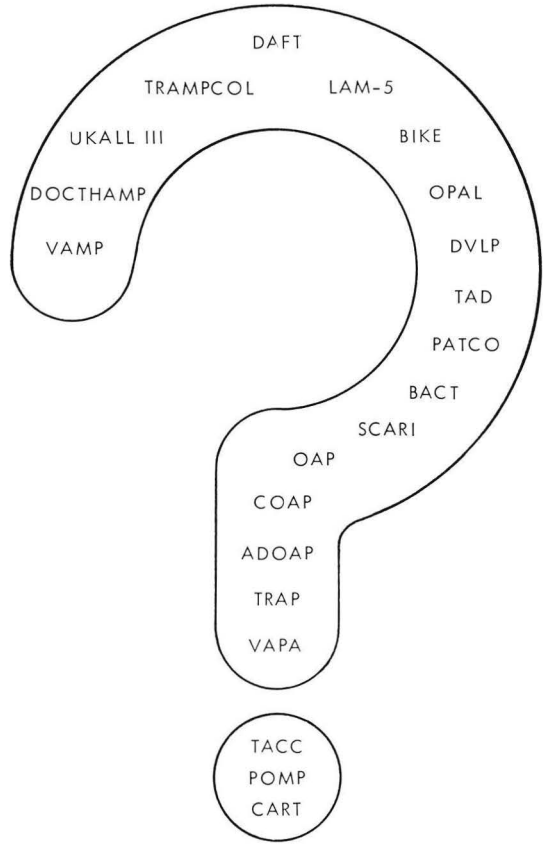


Figure 1.1:

"The" treatment of acute myeloid leukaemia.

find the optimal combination of cytostatic drugs and the most effective schedule of their administration. This approach has led to the presently widely used combination of Adriamycin and 1-β-D-Arabinofuranosyl-cytosine (ara-C). Still, a wide divergency can be observed among several clinical protocols as far as the interval-scheduling, the dose and the order of the two drugs are concerned (Carter et al., 1977). In certain solid tumours, Adriamycin has also become a valuable cytostatic drug, especially in breast cancer (Bonnadonna, 1977). As with myeloproliferative disorders in general a uniform dose is administered to all patients, consisting of 30-75 $mg.m^{-2}$. No information is available on the pharmacokinetic rationales behind this dosage, which may cause severe myelosuppression. Both the haematological and cardiac toxicity of Adriamycin limit its therapeutic usefulness, especially for prolonged administrations. Studies have been undertaken to investigate whether the distribution kinetics of the drug may explain its severe toxicity (Benjamin, 1975; Creasey et al., 1976). At present, it seems that Adriamycin accumulates extensively in various organs and is slowly eliminated from the body. Unfortunately, no information exists on pharmacokinetic bases as far as bone marrow toxicity is concerned. This demonstrates the need for extensive studies on the distribution and elimination of the drug when administered in current quantities and for a determination of whether the normal kinetics are modified in individuals with neoplastic disease. This last question, which is currently not under investigation neither with Adriamycin or with other drugs, may account for the well-known individual variation of toxicity and response to chemotherapy.

Although several experimental studies have yielded some information on the distribution kinetics of Adriamycin, they have not led to modifications of the clinical schedule of administration, probably due to problems concerning the extrapolation of animal data to the human situation. Moreover, data from humans often cannot be easily obtained. To minimize extrapolation problems, mathematical simulation models may serve to investigate (dis)similarities between animals and man, and even to predict distribution kinetics in the latter species. Mathematical simulation models are generally developed from animal data and applied to humans with consideration of species differences. Until now, however, most lack specificity due to substantial problems in solving their mathematics.

The problems outlined concerning the pharmacokinetics of Adriamycin prevent at present to give answers to the question of what is the optimal extracellular concentration of Adriamycin in both normal and tumour bearing organs and how should it be established. Since this

question is probably different for each type of tumour, a universal answer cannot be given. In view of the valuable activity of Adriamycin in acute myeloid leukaemia (AML), it has been the goal of this study to investigate some pharmacokinetic characteristics of Adriamycin in relation to this disease. As a model, the acute (pro)myelocytic leukaemia in the BN rat (BNML) has been used because its unique characteristics are very similar to those of human AML. Major advantages of the BNML are that the growth pattern and the response to chemotherapy are similar to that in AML (Hagenbeek, 1977; Aglietta and Colly, 1979; Aglietta and Sonneveld, 1978). After an introduction to the pharmacology and pharmacokinetics of Adriamycin (Chapter 2) the distribution kinetics of the drug in normal and BNML bearing animals will be described (Chapter 3). Attention will be paid to the question of whether the pharmacokinetics of Adriamycin are modified by the presence of tumour cells, which possibly show a different affinity to the drug. Therefore, in Chapter 4, some *in vitro* experiments will be described, which deal with dose-effect relations of Adriamycin in haemopoietic and leukaemic cells, such in view of the narrow space *in vivo* between leukaemic cell kill and toxicity to normal tissues. Chapter 5 will present a discussion of the development and application of a mathematical model for the *in vivo* distribution of Adriamycin in the rat. The reasons for the development of a mathematical model for the analysis of drug kinetics will be discussed in terms of the present lack of adequate information concerning cytostatic drugs. This concept is designed in a different way when compared with current models, since its aim is not to simulate distribution kinetics but to use general pharmacokinetic data to predict drug concentrations in inaccessible but important areas of the body. The relevance of the model for future clinical applications will be discussed. In Chapter 6, the value of each area of pharmacokinetic research, i.e., *in vivo* distribution, *in vitro* action and mathematical analysis, will be discussed and where possible connected.

CHAPTER 2

BIOCHEMISTRY AND PHARMACOLOGY OF ADRIAMYCIN

2.1 BIOCHEMISTRY AND MODE OF ACTION

Adriamycin is an anthracycline antibiotic isolated from surviving colonies of Streptomyces peucetius var. caesius after mutagenic treatment of the parent culture with N-nitroso-N-methyl-urethane. The drug is found mainly in the mycelium and is extracted with a mixture of acetone and aqueous sulfuric acid (Armacone et al., 1969). From a chemical point of view, Adriamycin is constituted of a reducing amino sugar (daunosamine) joined to an aglycone (adriamycinone) by a glycoside band. The structure and stereochemistry of Adriamycin are shown in Fig. 2.1.

The aglycone differs from the corresponding aglycone of Daunomycin, a related anthracycline antibiotic, only by the substitution of a hydrogen atom (on carbon-14) of the acetyl radical by a hydroxyl group (Armacone et al., 1969). Two major centers of physicochemical activity dominate the molecule. The planar anthracycline resonating ring system

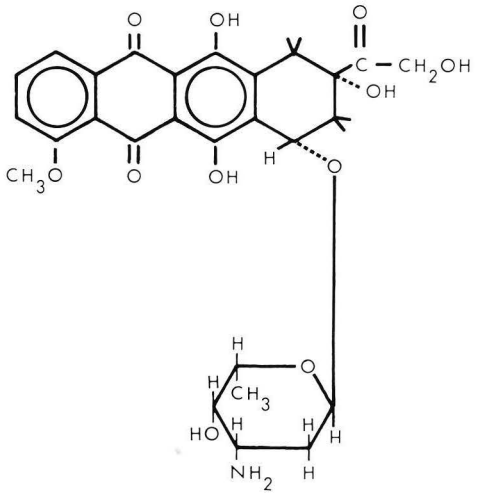


Figure 2.1:

Molecular structure of Adriamycin (Doxorubicin).

contains abundant hydroxyl groups adjacent to the amino sugar, which produces a hydrophilic center. If the amino sugar is removed, the resulting adriamycin aglycone is water insoluble, indicating the contribution of the amino sugar to compatibility with aqueous systems. In addition to its lipophilic and hydrophilic characteristics, the molecule is amphoteric and contains both acidic and basic functions in the phenolic groups and the sugar aminogroup, respectively (Bachur, 1975). Both the polarity and the charge of the molecule determine its affinity to numerous biological structures. In vivo, remarkable effects have been observed on the DNA dependent DNA and RNA synthesis, which may be related to the capacity of Adriamycin in vitro to bind specifically with DNA by intercalation between adjacent base pairs of the double helical structure (Zunino et al., 1972; DiMarco et al., 1971). This binding inhibits the enzymes involved in DNA replication (DNA-dependent DNA polymerase) by the induction of template disordering (DiMarco 1973). This inhibition of nuclear polymerases results in replication disorders which are manifested by death of cells in the S-phase of the cell cycle and by the induction of a block of cell cycle progression in the G_2 phase (Barlogie et al., 1975). The severity of the block may be a function of concentration and time.

The hypothesis that the biological effects of Adriamycin are related to the formation of a stable complex with DNA is strengthened by the observation that, in a series of anthracyclin analogs, there is a close relationship between the inhibitory effect on nucleic acid synthesis in vitro, inhibition of proliferative activity and DNA binding as measured by physicochemical methods (Zunino et al., 1972; Casazza et al., 1973); this will be further explained in Chapter 4.

2.2 METABOLISM

Most of the metabolism of Adriamycin occurs intracellularly. The cytoplasmic enzyme aldo-ketoreductase is the major catalytic agent in converting the drug to Adriamycinol (Fig. 2.2) (Bachur et al., 1974). An aldo-keto reductase has been isolated by Felsted from rat liver and purified to homogeneity (Fested et al., 1974). This enzyme has a molecular weight of 39,500 and consists of a single peptide unit. NADPH is a necessary cofactor for its function. The enzymatic product Adriamycinol retains an inhibitory activity against DNA and RNA synthesis (Bachur et al., 1976; Meriwether et al., 1972). This observation may be explained by the only small alteration of the D ring, which apparently does not affect the interaction with DNA.

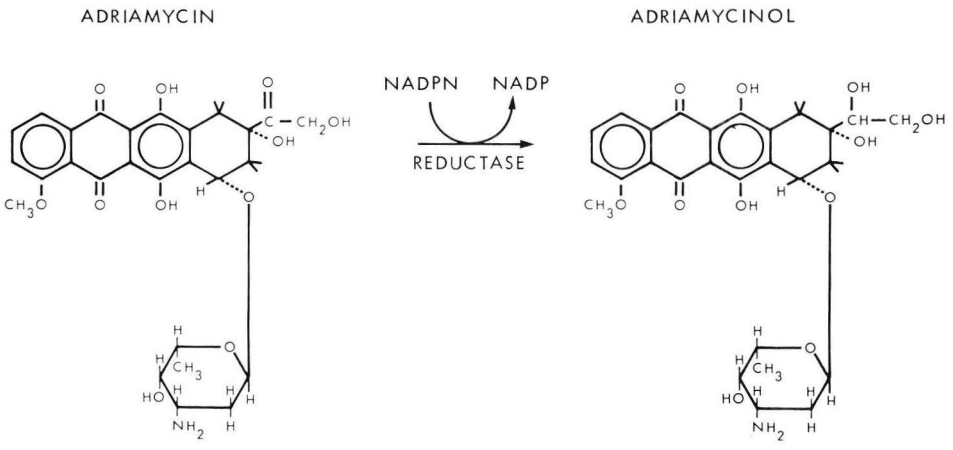


Figure 2.2:

Major metabolic pathway for the conversion of Adriamycin to Adriamycinol. (From: Bachur, 1975).

Two other major enzymes are involved in the metabolism of Adriamycin and Adriamycinol. A reductive glycosidase and a hydrolytic glycosidase remove the amino sugar from the anthracycline (Bachur and Gee, 1972; 1976). The reductive glycosidase, a microsomal enzyme, is inhibited by oxygen and is found in most tissues, especially in the liver (Bachur and Gee, 1971). The glycosidases yield products which have little activity against nucleic acid metabolism and are not cytotoxic. The aglycone products of the glycosidases are demethylated at the ring 4 position to yield demethyl-deoxyadriamycinol aglycone (Adriamycinone) (Bachur, 1975). The major steps in the metabolic pathway of Adriamycin are shown in Fig. 2.3.

In vitro, all organs except rabbit liver and kidney show very high glycosidase activities in the microsomes. However, only very moderate concentrations of aglycones, products of glycosidases, are detected in vivo in tissues, bile and urine (Bachur et al., 1973; 1974; Takanashi and Bachur, 1974). These data suggest that the metabolism of Adriamycin in vivo is mainly limited to the aldo-keto reductase activity, which produces the active metabolite Adriamycinol.

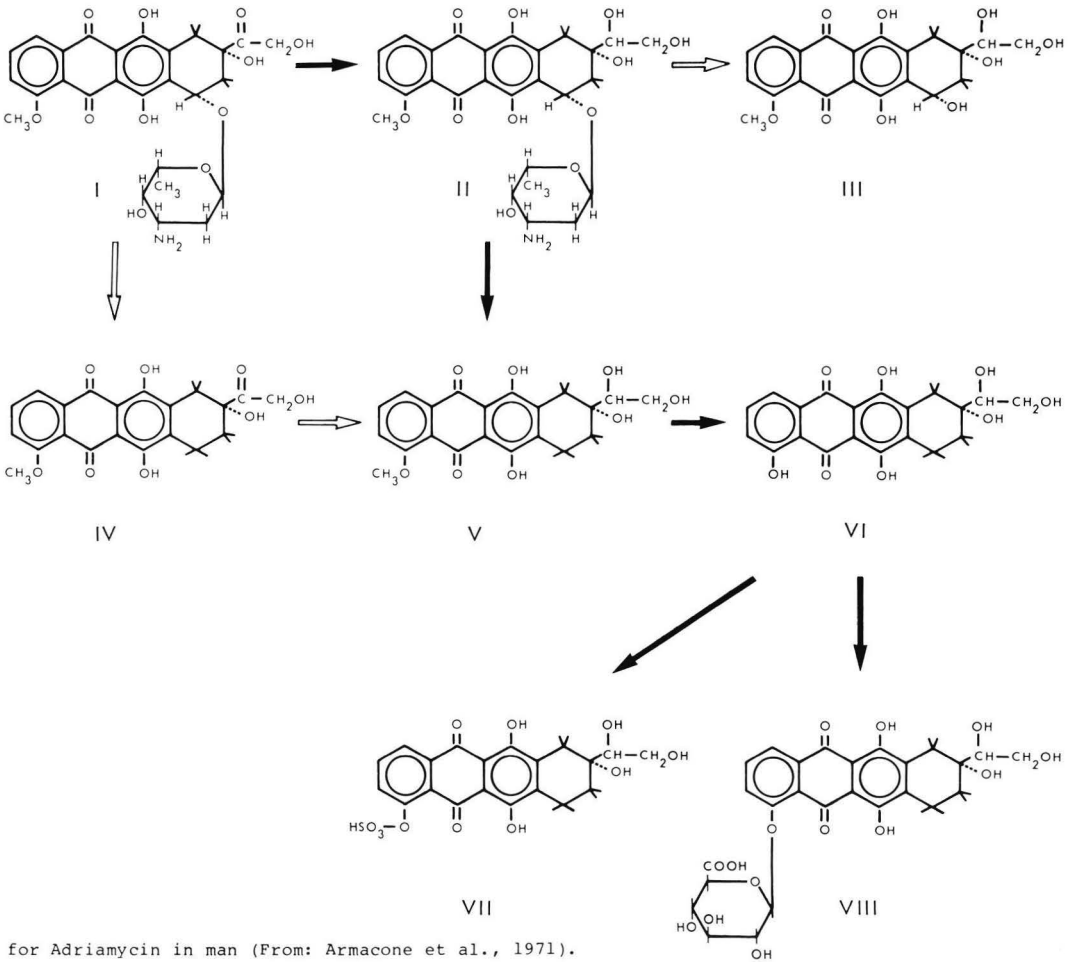


Figure 2.3:

Metabolic pathway for Adriamycin in man (From: Armacone et al., 1971).

2.3 PHARMACOLOGY

2.3.1. Determination procedures

The pharmacology of Adriamycin has been studied in a variety of animals and man. A common finding of these investigations appears to be that Adriamycin when administered i.v. is rapidly cleared from the plasma (Finkel et al., 1969; Bachur et al., 1970; 1976; Dusonchet et al., 1971; Yesair et al., 1972; Schwartz, 1973; Benjamin et al., 1973; Chan and Harris, 1973; Benjamin, 1975). In several investigations, the fluorescent property of the drug has been used in attempts at characterization, using a technique described by Kohn for identification of tetracyclines (Kohn, 1961). Dusonchet and co-workers described two emission peaks, at 540 and 584 nm, when Adriamycin dissolved in methylalcohol was excited at 465 nm (Dusonchet et al., 1971) (Fig. 2.4).

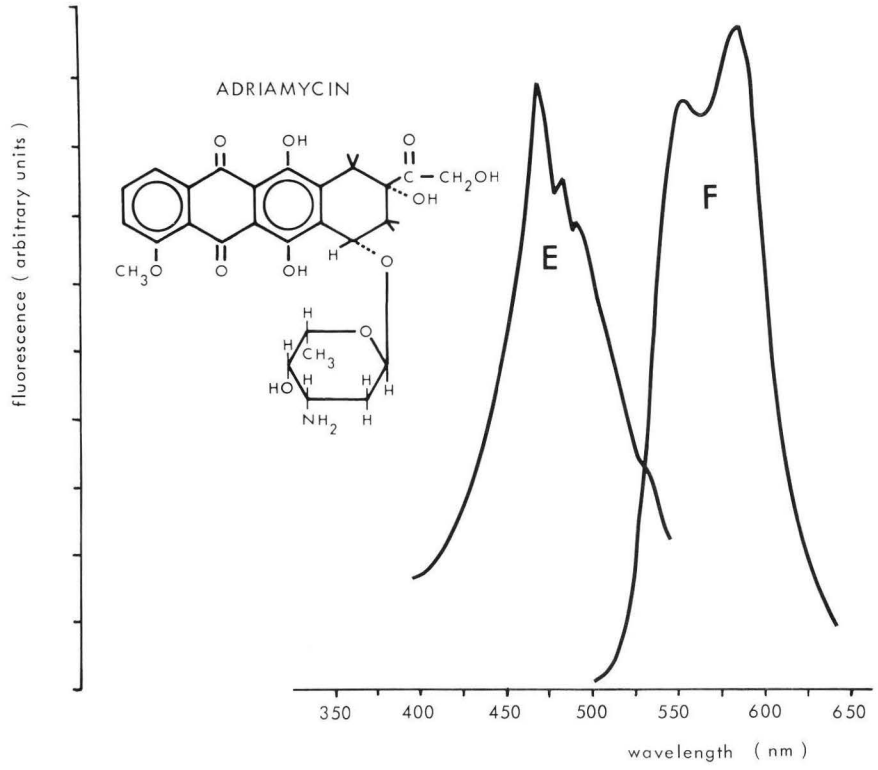


Figure 2.4:

Fluorescence spectra of Adriamycin; E = excitation spectrum; F = fluorescence spectrum.

This spectrofluorescent characterization of Adriamycin, which was linear for different concentrations, together with the reproducibility and the high sensitivity (0.01 µg per gram of tissue) led to the use of this technique for pharmacokinetic studies. However, in most investigations, the extraction method, required to break the protein bound Adriamycin and to produce a free solution of the drug, is different. The most commonly used extraction method has been described by Bachur et al. (1973), who employed a 50% mixture of HCl and ethanol to extract the protein bound Adriamycin. This procedure has a major limitation: it is reported to hydrolyze Adriamycin to its aglycone, thereby producing false metabolites which were not present originally in the sample. Furthermore, this method yields high tissue blank values and this limits its use in low concentration samples (Bachur et al.,1970).

The extraction procedure described by Schwartz is based on extraction with isoamyl alcohol in plasma, urine and tissue samples after release of the DNA-bound Adriamycin by Silver Nitrate (Schwartz, 1973). This method has the advantage of low tissue blank values, does not produce hydrolization of the parent drug and results in a high recovery factor.

Distribution studies of Adriamycin reported in the literature have been performed by using both methods. Therefore, the results are not completely comparable as far as metabolism of the drug is concerned and should be interpreted cautiously with respect to distribution patterns of the parent drug (Wilkinson, personal communication). Additional studies have been performed by several authors with other determination procedures. Bachur described a radioimmunoassay which was developed for situations in which the fluorescence assay is not available (Bachur et al., 1976). This method does not discreminate between Adriamycin and its active metabolite Adriamycinol. In contrast to fluorescence assays other fluorescent metabolites are probably overlooked. When compared with the fluorescence assay, a good correlation could be obtained only in patients with normal liver functions. Although possible false metabolites measured in the fluorescence assay could account for the lack of correlation in patients with hepatic malfunction, these metabolites other than Adriamycinol and its first breakdown product Adriamycinone should still be isolated, characterized and tested for their fluorescence behaviour as well as for cross reactivity in the radioimmunoassay before the merits of the two methods can be assessed.

Tritiated Adriamycin has been used by DiFronzo and co-workers to detect excretion pathways and penetration in the central nervous system (DiFronzo et al., 1973). Although these studies yielded some

important information, the interpretation of their data with regard to plasma drug levels and urinary excretion is complicated by difficulties in interpreting the significance of the levels of tritium measured. Bachur et al. (1970) have shown that, for the related anthracycline Daunomycin, the levels of radioactivity remained high while the levels of fluorescent materials closely related to the native drug fell progressively. The long plasma half-life of Adriamycin in the study of DiFronzo resembles that of tritiated water, which might be the result of exchange of radioactivity in the body fluids (Benjamin, 1975).

Eksborg recently described an analytical procedure for Adriamycin and Adriamycinol based on extraction with a mixture of chloroform and 1-pentanol as the organic phase, followed by reversed-phase high performance liquid chromatography using acetonitrile-water as the mobile phase. Quantitation was based on peak area measurements by photometric or fluorometric detection. An advantage of this method is the high recovery and the sensitivity (2 $ng.ml^{-1}$) (Eksborg, 1978). However, the equipment required to make these determinations is expensive and demands specially trained technicians.

As concluded from the variety of methods described in the literature, there is no agreement on the optimal assay for Adriamycin determinations in biological fluids. It is clear that, based on the high recovery factor obtained in tissues with the method of Schwartz, tissue concentrations can be determined most easily without risks of hydrolyzation of the parent drug. If necessary, separation procedures designed to make a distinction between metabolites and the parent drug can be included in the determination procedure. Several techniques have been reported to serve this purpose, including thin layer gel chromatography (Schwartz, 1973; Watson and Chan, 1977; Benjamin et al., 1973). An advantage of this chromatographic method is the relatively simple technical equipment needed to achieve separation of the metabolites from the parent drug.

2.3.2. Distribution in vivo

The physiological disposition of Adriamycin has been studied in several animal species and in man. The first to report on the pharmacokinetics of Adriamycin were Arena and co-workers, who studied the drug in mice and rabbits (Arena et al., 1971). Using a fluorometric method, they demonstrated that the drug was extensively incorporated into tissues. The tissue concentrations, which remained constant from

4 to 24 hours, were at least one or two orders of magnitude greater than blood levels in most organs studied. Later, Yesair et al. (1972) and DiFronzo et al. (1971) obtained similar results, although they used total fluorescence and ^{3}H-labelled Adriamycin, respectively. A major finding of these investigators was the primary route of excretion, which is via the bile. In all of these studies, it was shown that the uptake of Adriamycin in most tissues in mice appeared to be greater than the uptake of Daunomycin. The authors suggested that this differential uptake was responsible for the higher in vivo cytotoxicity and greater antitumour activity at equal doses of Adriamycin. However, their determination procedures were not always accurate enough to make a distinction between the different fluorescent compounds as to parent drug and metabolites. Adriamycin was cleared from the plasma rapidly, accumulated in tissue and excreted slowly into bile and urine in mice and rats. Major accumulation of Adriamycin took place in kidney and heart in rats and mice, while high concentrations were also detected in the lungs (Yesair et al., 1972). Studies by the same authors in dogs confirmed these findings and also showed high peak values in lungs.

It has been shown that Daunomycin and Adriamycin are extensively metabolized in mice, rabbits, hamsters, dogs and humans (DiFranzo et al., 1971; Wilkinson et al., 1979; Benjamin, 1973). In rats on the contrary no metabolism of Adriamycin could be demonstrated (Wilkinson et al., 1979).

The crucial results of these studies were confirmed by Bachur et al. (1974) in rabbits. However, they found both Daunomycin and Adriamycin to be extensively metabolized. There was no consistent striking difference in tissue concentrations of the two unchanged drugs, although most organs contained larger amounts of fluorescent Daunomycin metabolites than fluorescent Adriamycin metabolites. Some of these findings, particularly regarding the extensive metabolism, cannot be compared with those of other investigators because of the chemically aggressive extraction procedures used by Bachur et al. (See 2.3.1.).

The disposition of Adriamycin in man has been extensively studied by Benjamin et al. (1973; 1974), Bachur et al. (1973; 1975) and DiFronzo et al. (1973). Their clinical findings were in agreement with the animal studies performed earlier. Also in man the rapid disappearance of the drug from the plasma suggests a large volume distribution; in other words, the drug is widely dispersed in the tissues. In these patients, only 5% of the administered drug could be demonstrated in the urine within 5 days using fluorometric determination procedures. When tritium labelled Adriamycin was administered, 50% of the

radioactivity was excreted in 7 days following a dosage of 5 mg.kg^{-1}. Metabolites (Adriamycinol and Adriamycinone) frequently occurred in these patients, according to Benjamin et al. (1973) and Bachur (1975). They both used the fluorescent properties of the drug for determination of the plasma levels and showed that Adriamycin was predominantly metabolized in the liver to Adriamycinol and several aglycone derivates. Except for some "postmortem" examinations, extensive tissue determinations of Adriamycin have never been made (Chan et al., 1978). Therefore, no data are available on the in vivo distribution of Adriamycin in man. If judged only by the triphasic plasma disappearance curve a rapid distribution phase can be detected after administration of the drug; this is followed by a long, although quite variable, second phase. The terminal phase probably represents a recirculation of Adriamycin, which might be attributed to the enterohepatic recirculation. Except for the triphasic disappearance curve, which was originally thought to be only biphasic in animals, there seem to be no striking differences between the pharmacokinetics in man and animals (Chan et al., 1978). No information is yet available on the selective accumulation of Adriamycin in human organs, as demonstrated in animal models for spleen, liver, kidney and heart. This information can be obtained only by estimation procedures which relate the plasma concentration to drug levels in the rest of the body, which is theoretically divided into one or two compartments. These theoretical models for prediction of drug concentration will be discussed in Chapter 5.

In conclusion, one can say that Adriamycin is rapidly cleared from the plasma of all species, selectively accumulated in some organs (namely, the well-perfused tissues) and is excreted after a long period (e.g., days) via the biliary and the urinary routes. Dependent on the method of determination of the concentration in biological fluids and tissues, a variable extent of metabolic breakdown of the parent drug is found. The major detectable metabolite is Adriamycinol, the first breakdown product, of which the antitumour effect approaches that of Adriamycin. Another subject of considerable interest concerns the differential distribution of Adriamycin in primary and secondary tumours. Donelli et al. (1977) investigated concentrations of several anticancer drugs, including Adriamycin, in primary tumours (Lewis lung carcinoma, the sarcoma 180 in mice and the Walker 256 carcinosarcoma in rats) and in their lymph node metastases. It was shown in these three tumour models that the concentration of Adriamycin was significantly higher in pulmonary and lymph node metastases than in the parent tumour. This discrepancy may be due to proliferative characteristics, which often differ between primary and secondary tumours as

well as to differences in blood flow into the tumours, which is markedly dependent on the degree of necrosis. In general, blood flow into tumours is not easy to quantify in the individual patient. However, it can be quantified in animals and studies are now in progress to investigate its role in the tumour sensitivity to chemotherapy (Donelli, personal communication).

2.4 IN VITRO EFFECTS

In 1970, Rosella Silvestrini and co-workers (Silvestrini et al., 1970) described preliminary in vitro experiments with Adriamycin in comparison with the already known Daunomycin. They found that Adriamycin penetrated quickly into isolated Hela cells, giving rise to a severe reduction in the mitotic index after two hours. Autoradiographically, a 50% inhibition of DNA and RNA synthesis was observed at a dose of 5 $\mu g.ml^{-1}$ of Adriamycin; this inhibition was equal to that observed with Daunomycin. In the lower dose range, Adriamycin was found to be more active. These early results indicated what would become very clear. Adriamycin, which differs from Daunomycin only by the replacement of an H-atom by an OH-group, is more active against many neoplasms than its sister drug. However, the mode and mechanism of action is believed to be the same (DiMarco, 1975). Therefore, some basic experiments performed with Daunomycin can serve to reveal the mechanism of action of Adriamycin. Many authors have reported the inhibitory effect of Daunomycin on DNA and RNA polymerase reactions (Hartmann et al., 1964; Koschel et al., 1966; Bosmann and Kessel, 1971; Chandra et al., 1972; Zunino et al., 1974). The drug induced decrease in polymerase reactions is not the result of direct inhibition of DNA polymerase activity by Daunomycin, as was demonstrated in HeLa cells (Kim and Kim, 1972). The question remains as to what extent the inhibitory effects on nucleic acid synthesis are responsible for the inhibition of cell proliferation. Using doses of Daunomycin which do not affect DNA and RNA synthesis, an antimitotic effect has been observed in HeLa cells, even in the G_0 and G_1 phases of the cell cycle in which nucleic acid synthesis is not occurring (Tobey, 1972; DiMarco, 1975). Selective damage to the chromosomes is probably more relevant for the cytocidal effect than a quantitative reduction in the amount of DNA synthesized (Kim and Kim, 1972). Furthermore, it has been reported that in isolated rat fibroblasts the effect of Daunomycin on DNA synthesis is maximal during the late S phase, when the chromatin and centromers are replicated (Silvestrini et al., 1970).

The hypothesis that the effects of Adriamycin and Daunomycin are related to the formation of a stable complex with DNA is strengthened by the observation that there is a strict relationship between the inhibitory effect on nucleic acid synthesis and on proliferation, on the one hand, and the DNA binding capacity on the other (Zunino et al., 1972; DiMarco et al., 1973). In cultured mouse embryonic fibroblasts, DiMarco (1975) observed that a comparable inhibition of DNA synthesis was obtained with an intracellular concentration of Adriamycin that was 25% to 50% that of Daunomycin. These results were confirmed in L1210 cells, where the inhibitory effect of Adriamycin on DNA polymerase reactions in vitro was greater than that of Daunomycin (Meriwether and Bachur, 1972).

Several authors have claimed that the binding of anthracyclines to DNA is facilitated by the presence of DNA sequences rich in Adenine-Thymine (Necco, personal communication; Casazza et al., 1972; Zunino et al., 1975).

This hypothesis was supported by the fact that the inhibition of the DNA polymerase from RNA tumour viruses by anthracyclines is dependent on the type of primer templates used in the assay system (Chandra et al., 1972). This could explain the selective activity of anthracyclines on different normal and tumour cell lines. In addition to the data summarized above, in vitro experiments in a CHO Chinese hamster cell line demonstrated that noncycling cells treated for 2 hours with Adriamycin were triggered into proliferation and, after resuspending in a drug free medium, traversed the G_1 phase and were only slightly delayed in completing S and G_2. However, cycling populations traversed the S and G_2 very slowly, leading to an accumulation in G_1 (Tobey et al., 1976). This effect lasts for more than 16 h after transfer of the cells to a drug-free medium. From these results, it appears that cycling cells are more sensitive to the action of Adriamycin than are noncycling (= resting) cells. This effect could be based on the attempts of cycling cells to replicate DNA, which is inhibited in the presence of Adriamycin.

The antitumour cell activity of Adriamycin in vitro is not directly correlated with its intracellular concentration. However, the period during which an effective concentration is present determines the duration of the antineoplastic activity. Moreover, the cell cycle phase in which cells are present determines the sensitivity to Adriamycin. The extent of antineoplastic activity of the anthracycline shows some relation with the number of Adenine-Thymine bridges in the DNA molecule.

2.5 EFFECTS IN VIVO

Since its introduction, Adriamycin has shown remarkable effects against several experimental and clinical neoplasms. Some experimental results are described in section 2.5.1, as far as they are of interest for knowledge of the mode of action of the drug. In section 2.5.2, the most prominent clinical results, including some data on the clinical toxicity of the drug, are summarized.

2.5.1. Experimental results

Significant activity of Adriamycin has been observed in several experimental animal models, especially in the Ehrlich ascites tumour in mice, a transplanted lymphosarcoma in mice, in the 180 sarcoma, the L1210 mouse leukemia and in the Oberling-Guérin-Guérin myeloma in the rat (DiMarco, 1972; DiMarco et al., 1969; Sandberg, 1970; Mantovani et al., 1976a; Vecchi et al., 1978). In comparison with the antineoplastic activity of Daunomycin, that of Adriamycin is greater in most of these animal models. According to the results published by Mantovani and co-workers (Mantovani et al., 1976b), this difference in efficacy should be attributed to differences between the two analogues in suppressive effect on macrophages. In this regard, it is noteworthy that in several syngeneic murine tumour models they confirmed that the chemotherapeutic advantage of Adriamycin over Daunomycin is reduced or lost in immunosuppressed hosts (Mantovani et al.,1976b). The mechanism on which this advantage is probably based is specifically the relative sparing of macrophages by Adriamycin.

Apart from the well-known toxicity of anticancer agents which are dose-limiting such as myelosuppression, stomatitis, nausea and vomiting and alopecia, Adriamycin exhibits a unique harmful effect, that of cardiotoxicity. Long term administration leads to transient electrocardiographic abnormalities and eventually to irreversible cardiomyopathy. No relation with previously existing cardiac failure can be demonstrated. If the cumulative dose is limited to 450 mg.m^{-2}, cardiomyopathy is rarely observed (Lenaz et al., 1976). Since the frequency of cardiomyopathy is markedly increased at a total dose above 550 mg.m^{-2}, patients in whom this dose level has been exceeded should be monitored. Among several diagnostic procedures including echocardiography, measurement of the systolic time interval and electrocardiography, endocardial biopsy at regular time intervals is the most valuable tool in predicting cardiotoxicity (Mason et al., 1978). As no valuable

antidote has been found for the human situation, cardiotoxicity still remains a the major limiting factor in treatment with Adriamycin for longer periods.

In mice and rats, the extensive application of Adriamycin therapy is limited by the emerging problem of cardiomyopathy (Zbinden et al., 1975; Meyers, 1977; Sonneveld, 1978). Although some encouraging results have been obtained in reducing this side effect still no derivate has been developed which has the same or better therapeutic effects and less cardiotoxic activity. In long term treatment, the use of only 5 $mg.kg^{-1}$ single dose leads to a high incidence of adenocarcinomas and fibroadenomas of the breasts in female rats; these data indicate that application of Adriamycin in experimental studies is accompanied by a high incidence of unwanted side effects (Solcia et al., 1978). In view of the reported carcinogenic properties of certain anti-tumour agents in humans, the role of Adriamycin remains to be monitored in this respect in humans (Sieber, 1977).

2.5.2. Clinical studies

Adriamycin has been demonstrated to be an active drug against a number of malignancies, from which a survey is given in Table 2.1. However, here only its efficacy in the remission-induction of acute myelocytic leukaemia will be discussed. Most presently employed regimens for AML include either Daunomycin or Adriamycin combined with Arabinoside Cytosin (ara-C) and Vincristine, Prednisone, 6-Mercaptopurine or 6-Thioguanine. Many reports confirm the high response rates obtained with an anthracycline and ara-C, which were originally reported by Yates et al. (Yates et al., 1973; Preisler et al., 1977; Cassileth et al., 1977; Gale and Cline, 1977). Typical regimens combined from one to three doses of Adriamycin or Daunomycin with a five-day course of ara-C. Later, more intensive remission-induction combined three daily doses of an anthracycline with seven-day courses of ara-C. Several groups have reported 60 to 85% remission rates with these intensive regimens (Weil et al., 1976; Rai et al., 1975; Holland et al., 1976; Glucksberg et al., 1975; Gale and Cline, 1977). A major disadvantage of intensive chemotherapy in AML, however, is the high incidence of infections and haemorrhage, due to myelosuppression.

At present there are not yet sufficient data to recommend a specific regimen for maintenance and consolidation chemotherapy in AML, although most regimens include Adriamycin or Daunomycin (for a review, see Gale, 1979). Also with maintenance chemotherapy the myelosup-

pressive effect of Adriamycin frequently is poorly tolerated, often leading to premature discontinuation.

Table 2.1

ANTITUMOUR ACTIVITY SPECTRUM OF ADRIAMYCIN

Responsive	Poorly or not responsive
Breast adenocarcinoma	Large bowel adenocarcinoma
Stomach adenocarcinoma	Malignant melanoma
Soft tissue and bone sarcomas	Ovarian adenocarcinoma
Bladder adenocarcinoma	Renal cancer
Prostate adenocarcinoma	Chronic leukaemias
Bronchogenic carcinoma	Squamous cell carcinoma of cervix
Testicular carcinoma	Squamous cell carcinoma (head and neck)
Thyroid carcinoma	Pancreatic adenocarcinoma
Pediatric solid tumours	Brain tumours
Malignant lymphomas	
Acute leukaemias	
Hepatoma	
Multiple myeloma	
Squamous cell carcinoma of esophagus	
Endometrial adenocarcinoma	

CHAPTER 3

PHARMACOKINETICS OF ADRIAMYCIN IN NORMAL AND LEUKAEMIC RATS

3.1 INTRODUCTION

As stated in Chapter 2, little information is available on the comparative distribution kinetics of Adriamycin between normal tissue and malignant tumours in vivo, although the studies of DiFronzo et al. (1973) give an indication that the drug penetrates less extensively into solid tumours. Especially in the case of leukaemia, a widely disseminated malignant disease, individual variation in drug kinetics could theoretically account for the clinically observed variety of drug responses. Since a uniform dosage regimen is employed in the clinical situation without consideration of the relative influence of the number of tumour cells involved, attention will be paid in this Chapter to tumour burden related deviations from the distribution kinetics of Adriamycin in normal rats.

The Brown Norway Myeloid Leukaemia (BNML) was chosen as a model. This acute myelocytic leukaemia originated in 1971 in a female rat of the inbred Brown Norway rat strain in the Rijswijk colony (BN/Rij) following 3 intravenous injections of 2 mg of 9,10-dimethyl 1,2-benzanthracene 100 days earlier. The leukaemia has since been maintained by transplantation of leukaemic cells directly or from cryopreserved batches. The characteristics of the BNML have been described extensively (Hagenbeek, 1977) and can be summarized as follows:

1) the net growth rate is slow;
2) it is cytochemically and cytologically identical to human acute myeloid (promyelocytic) leukaemia (AML);
3) its response to chemotherapy is similar to that of AML;
4) the mean survival time after i.v. inoculation of 10^7 leukaemic spleen cells is 22 days.

These characteristics have made the BNML a particularly suitable model for the study of proliferation kinetics and experimental treatment of acute leukaemia (Aglietta and Sonneveld, 1978; Aglietta and Colly, 1979). Therefore, the distribution kinetics of Adriamycin were investigated in normal and BNML rats, the latter at a stage which is comparable with that of the human AML patient at the time of clinical

admission (day 15 after intravenous transplantation of 10^7 leukaemic spleen cells) (Hagenbeek, 1977). At this stage, bone marrow, spleen and liver are heavily infiltrated by leukaemic cells and normal haemopoiesis is severely suppressed. Table 3.1 summarizes some biological characteristics of the BNML.

Table 3.1

DIFFERENT CHARACTERISTICS OF BN AND BNML RATS*

	BN	BNML**
Spleen weight	0.35 g	1.28 g
Number of BNML cells in spleen	0	9×10^8
Femoral bone marrow cellularity	10^8	5×10^7
Percentage BNML cells in femoral bone marrow	0	65
Liver weight	4.94	8.50

* at day 15 after inoculation of 10^7 viable leukaemic spleen cells. Values represent means of 5-40 measurements.
**(According to Hagenbeek, 1977).

3.2 GENERAL EXPERIMENTAL PROCEDURES

3.2.1. Animals

The studies were performed in the inbred Brown Norway (BN) rat strain from the Rijswijk colony. Female rats of 12 weeks of age, born and bred under specific pathogen-free (SPF) conditions, were used. Water and food were always supplied ad libitum during the experiments.

3.2.2. Experimental drug

Adriamycin hydrochloride was kindly supplied by Farmitalia, Milan, Italy. The chemical purity was assessed by thin-layer chromatography, using Merck silica gel glass discs. The solvent used was:
- normal butanol 60%;
- ice-cold acetic acid 15%;
- distilled water 25%.

The chemical purity of the drug was 99.5%. No variations in purity were noted during dry storage of the drug at room temperature.

3.2.3. Leukaemia transfer

The leukaemic spleen was always used as a source of leukaemic cells for transplantation. After resuspension of spleen cells obtained from a 21-day-old leukaemic rat in Hanks' balanced salt solution, the required number of cells was injected intravenously into a tail vein of the recipient in a final volume of 1 ml.

Distribution studies in leukaemic rats were always performed at day 15 after inoculation of 10^7 leukemic spleen cells.

3.2.4. Collection of biological specimens

Adriamycin hydrochloride was, after solution in sterile 0.9% NaCl at a final volume of 2 $mg.ml^{-1}$, administered into a tail vein at a dose of 7.5 $mg.kg^{-1}$ (= 40 $mg.m^{-2}$ of body surface). It was always dissolved 2 h before each experiment.

To determine the different concentrations of the drug in plasma and organs, animals were killed at various intervals after intravenous administration of the drug. For each time interval, 4 experimental and 1 control animal were used. Animals were anaesthesized by aether. After the abdomen was opened blood was obtained by aortic puncture, and sampled in heparinized tubes. The amount of blood obtained (generally 4 ml) was centrifuged for 10 min at 400 g. After carefully pipetting off the upper phase, plasma was kept at +4 °C until chemical analysis was performed. Liver, spleen, kidneys, heart and lungs were removed from the body, weighed and homogenized immediately after addition of sterile 0.9% NaCl to obtain a final dilution of 10% v/v. Immediately after the homogenation, the tissue samples were stored at 4 °C. Bone marrow was obtained after the femurs were cleaned of adherent muscle tissue; the proximal ends were cut with a surgical knife and the femoral shafts were emptied by repeated flushing with Hanks' solution using a needle placed on a 5 ml syringe. The collected bone marrow was filtered and cells were counted microscopically using a Bürker type haemocytometer after staining with Türck's solution (0.01%).

3.2.5. Extraction procedure

For deproteinizing the cell suspensions and extraction of serum and cell suspensions, the method described by Schwartz proved to produce the highest recovery of Adriamycin (Schwartz, 1973). As discussed in section 2.3.1., this method does not give rise of unwanted metabolization of the parent drug during the extraction procedure. However, some slight modifications were introduced to improve the recovery. Therefore, the modified extraction method is described.

Using automatic Excalibur pipettes, 0.4 ml of plasma was pipetted into silicated glass tubes and 0.3 ml distilled water was added. After addition of 4.0 ml isoamyl alcohol (Merck, 941), the tubes were stored for 10 min at 4° C to extract protein bound Adriamycin. Immediately after the extraction period, the samples were centrifuged for 20 min at 800 g. The supernatant was then collected and stored at 4° C in the dark until determination.

Cell suspensions of the different organs at a volume of 1 ml were added to 0.2 ml of 33% $AgNO_3$ (Lamers and Indemans, 's-Hertogenbosch, The Netherlands) and mixed for 10 min. This step in the procedure was essential for disruption of the cells and to allow deproteinization. After addition of 4 ml of isoamyl alcohol and mixing during 10 min at 4° C, the samples were centrifuged for 20 min at 800 g. The supernatant was collected and stored at 4° C.

Determination of the Adriamycin concentration was performed spectrophotometrically in a Hitachi-FL 204 fluorescence spectrophotometer. It proved to be necessary to heat the samples to 30° C before determination in order to prevent false measurements due to cold induced opacities of the cuvettes.

In all experiments, the excitation wave length was 460 nm and the samples were read at a fluorescence of 580 nm. To adjust the scaling of the spectrophotometer, an external standard solution of 10 µg Adriamycin per ml sterile water was compared with a stock solution of quinidine before each experiment. Pure isoamyl alcohol served as the blank control value. An internal standard was processed in each experiment, consisting of 10 µg Adriamycin dissolved in serum obtained from a frozen stock. With this internal standard, the recovery factor in serum can be assessed in each experiment and technical errors during the extraction procedure can be detected.

3.2.6. Renal function

Since Adriamycin is partly excreted via the urinary route, it is necessary to monitor the renal function in experiments concerning the pharmacokinetics of the drug. Especially in the case of leukaemic animals, where infiltration of the kidneys with leukaemic cells takes place, should determination of the renal function occur. In this study, the creatinine concentration of the serum, being a function of the renal excretion capacity, was ascertained in normal rats and in leukaemic rats at different stages of the disease. A standard commercial kit (Boehringer, Mannheim, W. Germany) was used for spectrometric determinations.

3.2.7. Distribution of ^{59}Fe

In view of the reported toxicity of Adriamycin to bone marrow cells, the concentration time history of Adriamycin in this tissue was investigated. Because of the easy accessibility of the femoral bone marrow all concentrations were determined in this section of the organ. The relative contribution of this part to total bone marrow was investigated using ^{59}Fe, because, in literature a wide variation in total bone marrow cellularity between rat strains has been reported (Pegg, 1966), with a mean cellularity of 29.4×10^{9} .kg^{-1} body weight in Wistar rats. To determine the total bone marrow cellularity in this rat strain, ^{59}Fe (spec. act., 100 µC.ml^{-1}; Radiochemical Centre, Amersham, England) was injected intravenously into 5 rats in a final concentration of 10 µC each. At 48 h, the rats were after a cardiac puncture to obtain blood killed by cervical dislocation and the liver, spleen, and right femur were removed. Bone marrow cells were obtained from the femur by cutting and repeatedly flushing the femoral shafts with Hanks' balanced salt solution. Spleen and liver cells were suspended by forcing tissue through nylon gauze. In cell suspensions of liver, spleen and bone marrow and in the peripheral blood, the gamma radioactivity emitted by the isotope, expressed as counts per minute (cpm), was measured in a scintillation counter (Model 4227, Nuclear Chicago Corp., Des Plaines, Ill., USA). The background radioactivity was subtraced from each counted sample.

The volume of the bone marrow compartment was calculated by subtraction of the radioactivity present in spleen, liver and peripheral blood, from the total radioactivity emitted by 10 µC of the isotope.

3.2.8. Plasma volume

The plasma volume was determined by injection of ^{14}C-Inulin (spec. act. 1-3 µCi.mg^{-1} of Inulin-carboxylic acid) at a final concentration of 10 µCi in 1 ml. After 1 min, a cardiac puncture was performed and the blood was sampled in tubes containing 150 IU heparin and centrifuged at 700 g for 10 min. The plasma was pipetted off and the radioactivity, expressed as counts per min, was measured in scintillation vials (Packard Instr., Zurich, Switzerland) in a liquid scintillation counter (Nuclear Chicago, Mark II) using a toluene based scintillation fluid (50 mg POPOP and 4 g PPO per liter toluene). The plasma volume was calculated by dividing the CPM produced by 10 µCi ^{14}C-Inulin by that produced by 1 ml of plasma. The total blood volume was derived from this value by multiplying the plasma volume by the reciprocal of the haematocrit.

3.3 RESULTS

3.3.1. Adjustment of internal standards

At several times during the study, internal standards were prepared by addition of 10 µg Adriamycin in a volume of 10 µl to a 1 ml serum volume, a 1 ml suspension of various organs or 10^8 cells of femoral bone marrow. After 1 h of incubation at 37° C, the fluorescence was determined. Before and after the study, a titration curve of these standards was prepared for calculation purposes. During the study, the fluorescence of these standards was constant, with a S.D. of less than 10%. The mean fluorescence is indicated in Table 3.2. Calculations of the drug concentrations in the various samples were made by using a standard curve composed from several concentrations. As an example, the standard curve for serum is shown in Fig. 3.1. Since these curves proved to be linear in all organs up to 150 µg Adriamycin per gram of tissue, unknown concentrations can be calculated from $C = \frac{F}{FI} \times CI$, where F is the observed fluorescence, FI is the fluorescence of the Internal Standard and CI is the concentration of Adriamycin in the Internal Standard.

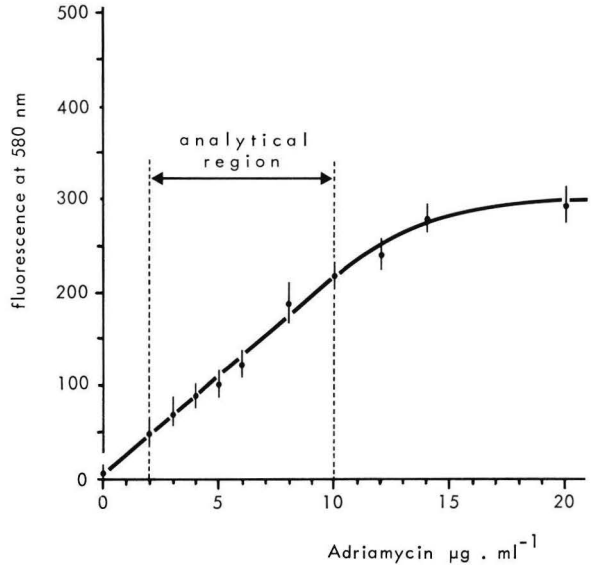


Figure 3.1

Calibration curve of different concentrations of Adriamycin dissolved in fresh serum. The symbols represent the mean values of 5 separate experiments (M ± SD).

Table 3.2

INTERNAL STANDARDS OF SERUM AND ORGANS (M ± SD)

	plasma	liver	spleen	heart	kidney	lung	bone marrow
normal	195.6	339.2	318.8	345.8	342.2	352.3	288.7
	12.1	24.1	28.7	31.0	34.0	10.6	12.1
15th day leukaemia	187.0	322.3	351.0	323.8	317.3	344.8	407.5
	13.1	20.3	28.1	26.8	28.5	44.8	19.2

Fluorescence of standard solutions of 10 ug Adriamycin in 1 ml serum or 1 ml of 10% cell suspension (w/v). Bone marrow fluorescence indicates the standard of 10 µg Adriamycin in 10^8 femoral marrow cells in 1 ml Hanks.

3.3.2. Distribution of Adriamycin in normal and leukaemic rats

To study the plasma disappearance curve of Adriamycin, normal and 15th day leukaemic rats were injected intravenously with single doses of various concentrations and plasma samples were analyzed at several intervals after drug administration. Fig. 3.2 shows the plasma curve after 7.5 mg.kg^{-1} in normal rats. Based on surface area conversion factors described by Freireich et al. (1966), this dose is comparable with the clinically applied dose of 60 mg.m^{-2}. Careful analysis of this curve reveals that the shape is triphasic, indicating that the distribution kinetics of the drug following this dose are complex. A first half-life of 35 min due to the rapid uptake into the tissues is followed by a long second phase which lasts for more than 16 h and can be attributed to very slow release of the drug from the tissues. The third phase in the curve cannot be explained by conven-

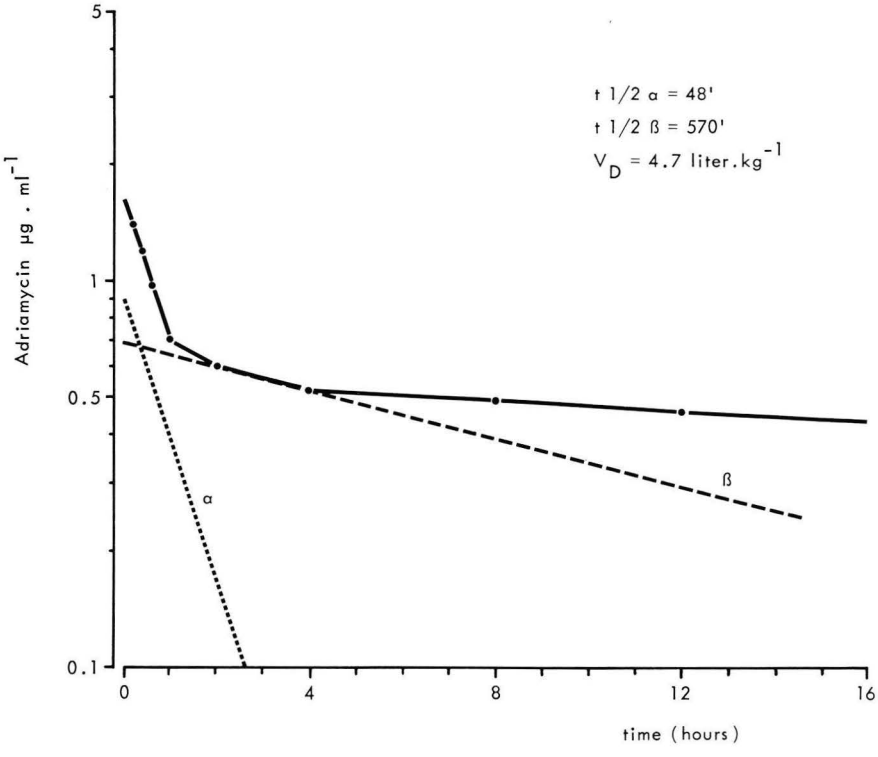


Figure 3.2

Plasma disappearance curve of Adriamycin following a rapid intravenous bolus injection of 7.5 mg.kg^{-1} dissolved in 0.9% NaCl in a final concentration of 2 mg.ml^{-1}. The symbols represent mean values of 8 animals per point.

tional pharmacokinetic assumptions but could theoretically be attributed to the possible role of the enterohepatic recirculation.

To study the linearity of this distribution, single doses of 3.75 $mg.kg^{-1}$ and 15 $mg.kg^{-1}$ were administered intravenously. Fig. 3.3 shows the plasma disappearance curves after the various doses. It can be concluded from the curves that a triphasic disappearance pattern is evident after 3.75 $mg.kg^{-1}$, 7.5 $mg.kg^{-1}$ and 15 $mg.kg^{-1}$. Little difference is observed between the two lower doses with respect to the maximum plasma level and the disappearance of the drug from the plasma. However, administration of the highest dose leads to prolonged, higher plasma levels. The shape of all curves is superimposable, which indicates that the *in vivo* distribution of Adriamycin is probably linear in this dose range (Wagner, 1975).

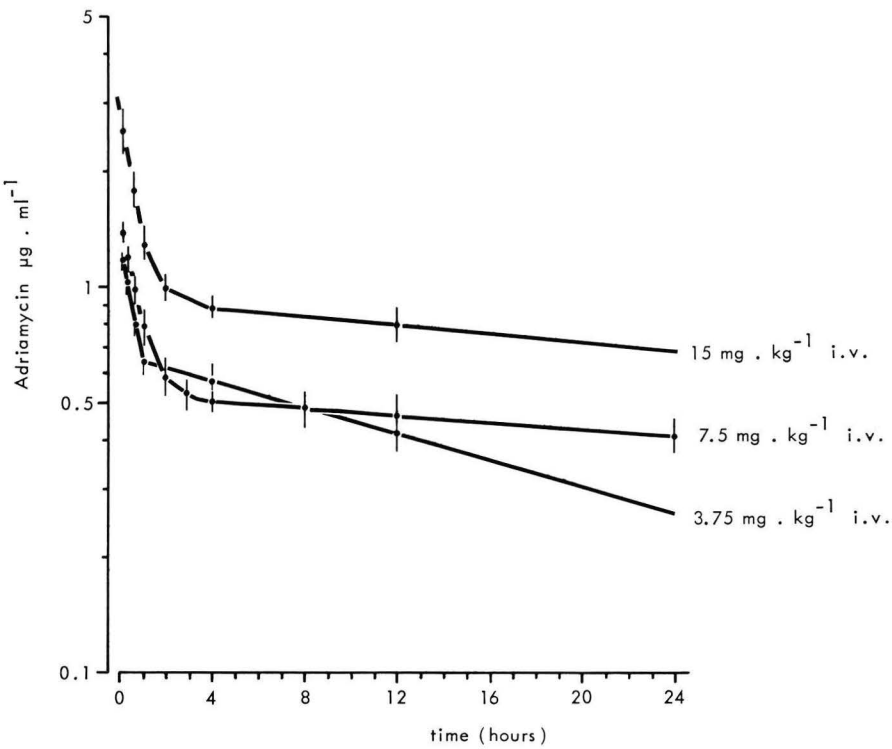


Figure 3.3

Plasma disappearance curves of Adriamycin following single rapid intravenous bolus injections of 3.75 $mg.kg^{-1}$, 7.5 $mg.kg^{-1}$ and 15 $mg.kg^{-1}$, dissolved in 0.9% NaCl to a final volume of 1 ml. The symbols represent mean values of 4 animals per point (M ± 2 SE).

Although the concentration time history in plasma provides information on the extent of drug penetration in the tissues, selective accumulation can be detected only by measuring the drug concentration in various organs. Since a leukaemia model was chosen to study the distribution of Adriamycin as a function of tumour load, the following organs were selected for examination: liver, spleen and femoral bone marrow (as major sites of leukaemic infiltration), heart and lungs (as major sites for selective toxicity) and kidneys because of their excretory function. Figures 3.4 - 3.11 show the typical disappearance curves obtained after intravenous administration of 7.5 $mg.kg^{-1}$ in normal and in leukaemic rats at day 15 of the disease. At this stage, the mean weights of the organs investigated average the values indicated in Table 3.3.

TABLE 3.3

WEIGHTS OF BODY AND ORGANS OF NORMAL AND 15-DAY LEUKAEMIC RATS

	Normal (g)	15-day leukaemia (g)
Body weight	165.05 ± 3.10	165.85 ± 4.55
Plasma	5.70 ± 0.18	5.10 ± 0.18
Spleen	0.35 ± 0.11	1.28 ± 0.10
Liver	4.94 ± 0.25	8.50 ± 0.38
Heart	0.60 ± 0.04	0.58 ± 0.02
Lungs	1.35 ± 0.02	1.38 ± 0.03
Kidneys	0.96 ± 0.01	1.04 ± 0.02
Skeleton	10.40 ± 0.24	10.45 ± 0.63
Skin	35.00 ± 1.45	34.00 ± 2.06

The results represent the values obtained in ± 60 female animals of 12 weeks of age (M ± SD).

Plasma (Fig. 3.4): As stated above, the plasma concentration after intravenous administration of 7.5 $mg.kg^{-1}$ disappears according to a triphasic pattern. Calculation of the extrapolated volume distribution from the experimental data gives an indication of the relation between the amount and the concentration of the drug and may be derived from:

$$Vd_{ext} = \frac{\text{dose/body weight}}{\text{plasma concentration at time } t = 0}$$

where Vd_{ext} stands for the extrapolated volume distribution. The

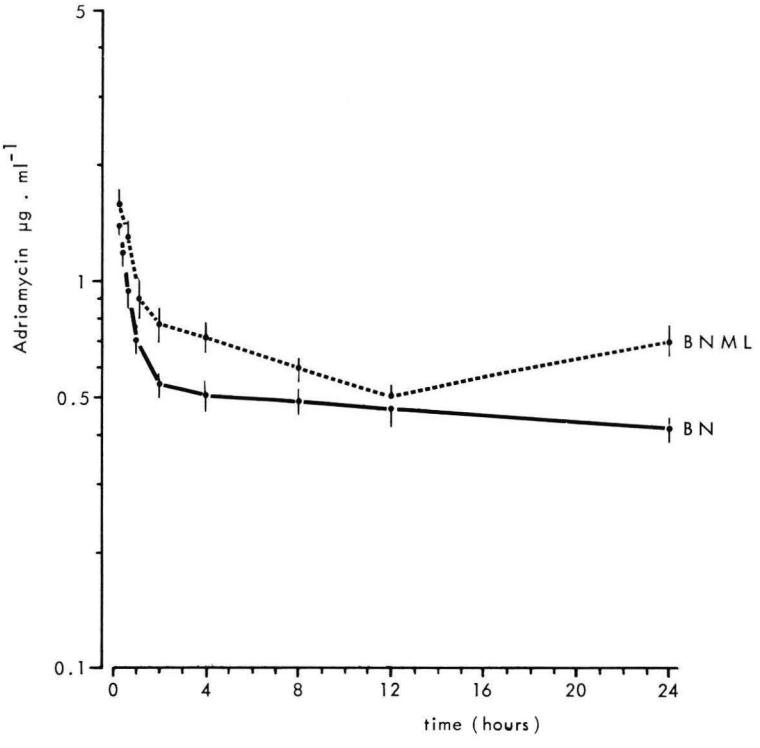


Figure 3.4

Plasma concentration of Adriamycin in normal and 15-day leukaemic rats following 7.5 mg.kg^{-1} intravenously (M ±2 SE). The symbols indicate the concentration per ml of plasma.

volume distribution of Adriamycin in the normal rat is 4.7 l.kg^{-1}; keeping in mind that an equal distribution through plasma and the rest of the body is represented by Vd_{ext} = 11 . kg^{-1}, this rather high value of 4.7.kg^{-1} implies that Adriamycin is extensively accumulated in the tissues. In comparison to this, the volume distribution in leukaemic rats is 3.9 l.kg^{-1}, suggesting that a less extensive accumulation in tissue occurs than in normal rats. Initially, the plasma concentration decreases more slowly in leukaemic rats, as can be concluded from the levels which are a factor of 1.3 higher in the early 60 min. This first disappearance phase also lasts for 30 min longer than in normal animals, leading to an initial half life of 62 min instead of 35 min. Therefore, the second phase of the disappearance curve, representing the excretion from the tissues to the blood, appears to start 60 min after injection and lasts for ± 30 h in leukaemic rats. The exchange

from the plasma to some tissues and the reverse has probably been decreased by leukaemic infiltration of those tissues. Twenty-four hours after drug administration, a detectable level is still present in both normal and leukaemic rats. It appeared that in leukaemic rats at 48 h after drug administration still a detectable concentration of Adriamycin ($0.3\ \mu g.ml^{-1}$) was present in the plasma (not mentioned in the figure), such in contrast to normal rats where at this interval no drug level was demonstrable. This suggests a very slow excretion in leukaemic rats.

Spleen (Fig. 3.5): Adriamycin concentrations in the spleen reach values which are 10-fold higher than the plasma concentration within 10 min after drug administration, in accord with the rapid decrease observed in the plasma. Despite the initial decrease in the first 30 min, the spleen drug levels remain high for 24 h in normal rats. In leukaemic animals in which the spleen weight has increased by a factor of 5

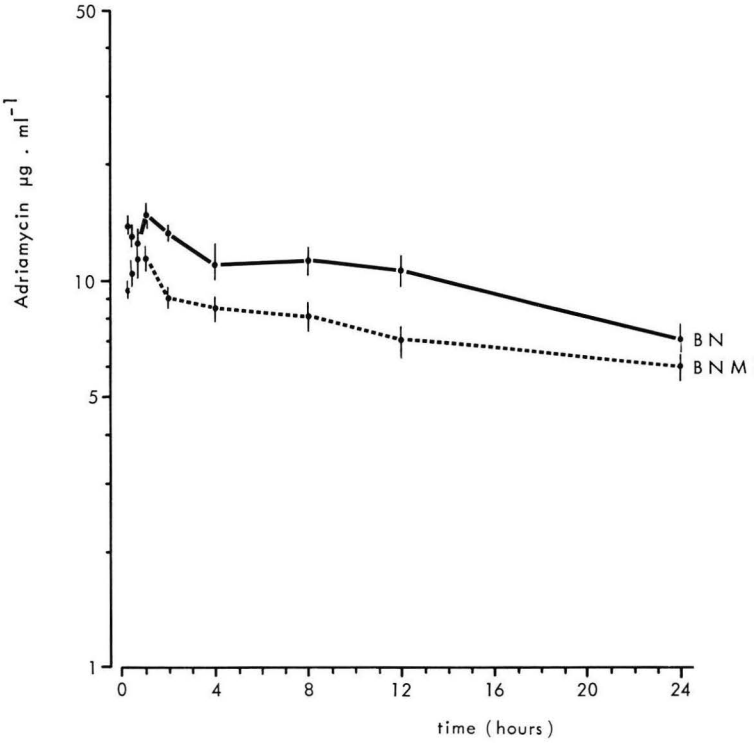


Figure 3.5

Spleen concentration of Adriamycin in normal and 15-day leukaemic rats following $7.5\ mg.kg^{-1}$ intravenously (M ± 2 SE). The symbols represent the concentration per gram of tissue.

and large numbers of leukaemic cells have infiltrated into the organ, the maximum drug concentration does not exceed 11.5 µg.g^{-1} of tissue. The drug levels are generally diminished by a factor of 2 in the first 24 h when compared with those of normal animals.

Liver (Fig. 3.6): Very high concentrations of Adriamycin are observed in the liver within 10 min. The disappearance curve shows a triphasic pattern which is typical for a well-perfused organ. Little difference can be observed between normal and leukaemic rats. As in the spleen, considerable drug levels are present in the liver for 24 h and even for 48 h.

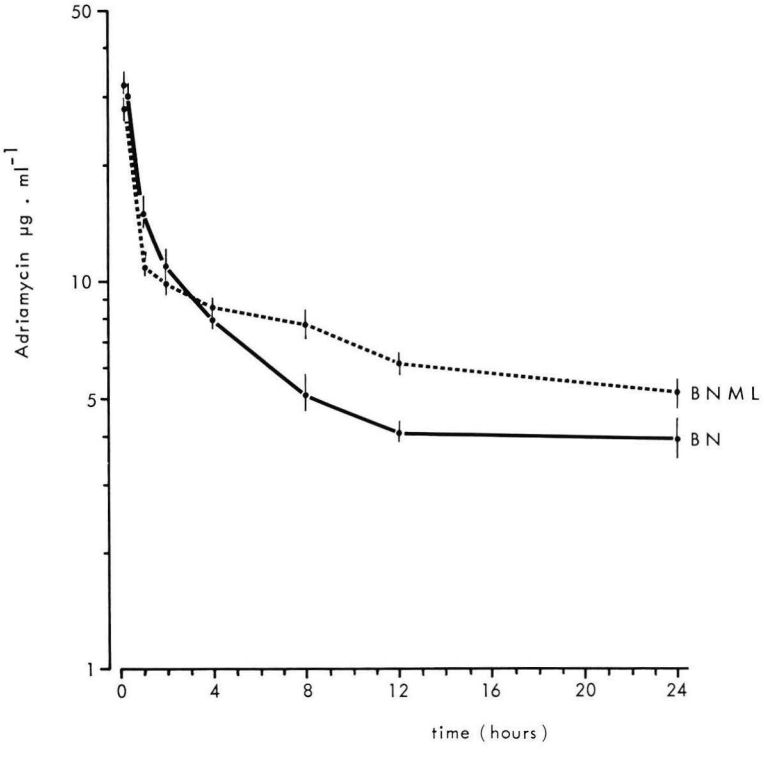


Figure 3.6

Liver concentration of Adriamycin in normal and 15-day leukaemic rats following 7.5 mg.kg^{-1} intravenously (M ± 2 SE). The symbols represent the concentration per gram of tissue.

Heart (Fig. 3.7): Analysis of the heart disappearance curve does not show abnormally high drug levels when compared with other organs. However, in this well perfused organ the slow decrease in the concentration of Adriamycin is remarkable. No striking deviations from this pattern are observed in leukaemic animals, as can be expected from the data of Hagenbeek (1977), who observed no leukaemic infiltration of the heart at day 15 of the disease.

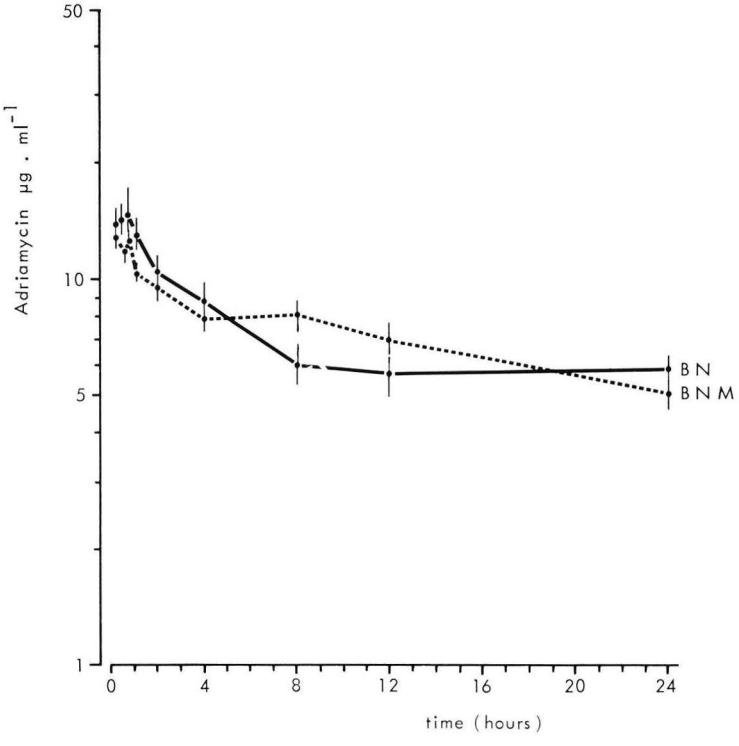


Figure 3.7

Heart concentration of Adriamycin in normal and 15-day leukaemic rats following 7.5 mg.kg^{-1} intravenously (M ± 2 SE). The symbols represent the concentration per gram of tissue.

Kidneys (Fig. 3.8): As is observed in the liver, very high concentrations of Adriamycin are present in the kidney, especially in the early phase after injection. Since the urinary route is a major elimination pathway, drug accumulation in kidney tissue is not surprising. The presence of a leukaemic cell tumour load in the body does not change the disappearance curve.

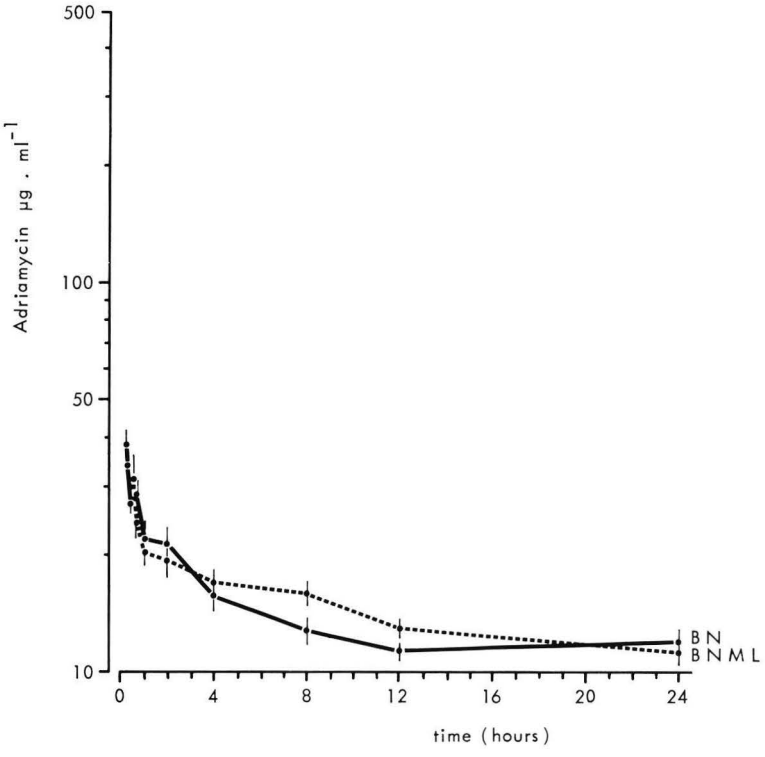


Figure 3.8
Kidney concentration of Adriamycin in normal and 15-day leukaemic rats following 7.5 mg.kg^{-1} intravenously (M ± 2 SE). The symbols represent the concentration in both both kidneys per gram of tissue.

Lungs (Fig. 3.9): As can be expected from the absence of leukaemic infiltration in the lungs (Hagenbeek, 1977), the disappearance curves of normal and leukaemic rats do not differ significantly in either shape or quantitative aspects at this stage of the disease.

Bone marrow (Fig. 3.10): Considerable concentrations of Adriamycin are observed in the femoral bone marrow of both normal and leukaemic animals. In Fig. 3.10, the concentration is plotted per 10^8 nucleated cells. Assuming a mean cell diameter of 8 µ and a specific density of 1.00, the weight of 10^8 cells is 26 mg. This indicates that the concentration of Adriamycin at 0.5 h after administration amounts to 144 µg.g^{-1} of bone marrow cells in normal animals. Although in leukaemic femoral bone marrow, the maximum concentration is only 50% of this value, there is still great difference between the concentration found

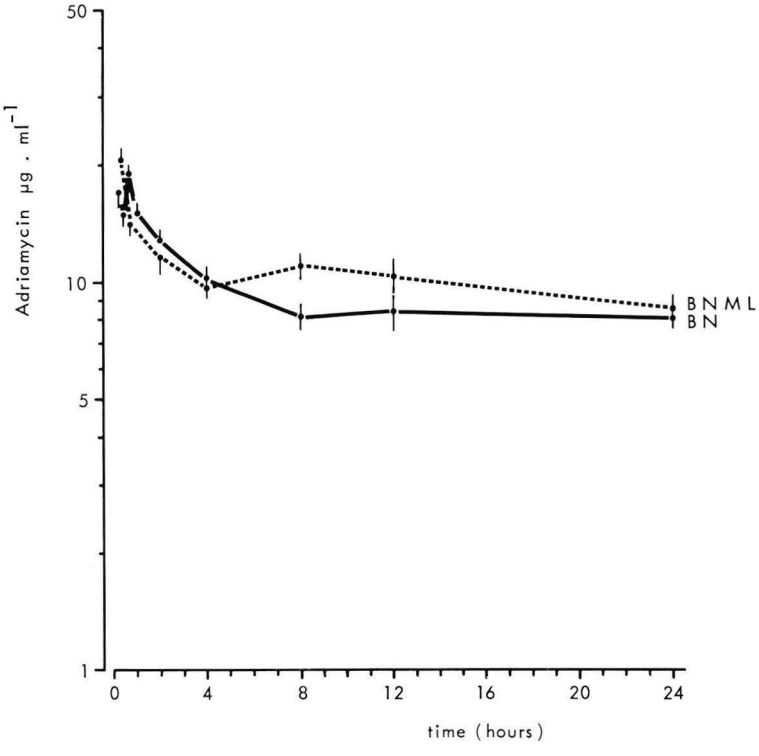


Figure 3.9

Lung concentration of Adriamycin in normal and 15-day leukaemic rats following 7.5 mg.kg^{-1} intravenously (M ± 2 SE). The symbols represent the concentration in both lungs per gram of tissue.

in bone marrow cells when compared with that in cells of other organs. To evaluate these differences in quantitative aspects, the total volume of the bone marrow was determined by using ^{59}Fe. An equal distribution of the isotope through the bone marrow was supposed and the radioactivity after subtraction of that in liver, spleen and peripheral blood was used to determine the relative contribution of the femur to the total bone marrow. It was found that one femur represents 2.5% of the total bone marrow in BN rats (Table 3.4); this means that 144 µg of the administered Adriamycin accumulates in normal bone marrow, i.e., 11.6% of the total dose at 30 min. Later the total drug concentration in bone marrow even increases, indicating that a major part of the administered dose accumulates in bone marrow cells. In the BNML rats, femoral bone marrow represents 1.2 % of the whole haemopoietic organ. Adriamycin also accumulates here, although to a somewhat

Table 3.4

DISTRIBUTION OF ^{59}Fe IN BN AND BNML RATS* AFTER INTRAVENOUS ADMINISTRATION OF 10 µCi** IN 1 ml.

	BN		BNML	
Organ	weight (g)	cpm x 10^2	weight (g)	cpm x 10^2
Spleen	0.42 ± 0.05	73.0 ± 3.6	1.28 ± 0.10	41.1 ± 15.2
Liver	5.32 ± 0.27	1134 ± 79.1	8.50 ± 0.38	533.1 ± 98.7
Peripheral blood	9.50 ± 0.21	3170 ± 213.8	9.50 ± 0.21	2537.2 ± 688.9
Femur	10^8 cells	21.1 ± 3.1	5x 10^8 cells	24.1 ± 2.1
Total bone marrow		813.0		2003.8
Femoral fraction of normal bone marrow		2.59%		1.21%

* At day 15 after i.v. transplantation of 10^7 leukaemic spleen cells.
**10 µCi = 5190 x 10^2 cpm.

lesser degree, but again progressively. This preference of the drug to accumulate in bone marrow cells probably explains its toxic effect on normal haemopoiesis and the remarkable reducing effect on the leukaemic cell population; this will be further discussed in Chapter 4, section 4.

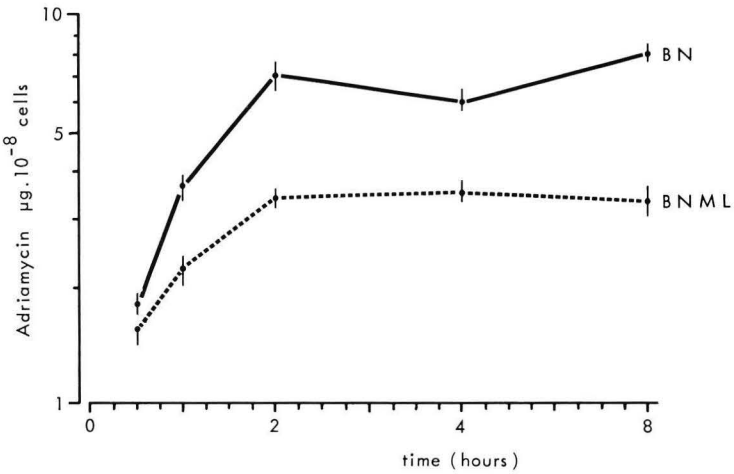


Figure 3.10

Concentration of Adriamycin in femoral bone marrow cells of normal and 15-day leukaemic rats following 7.5 mg.kg^{-1} intravenously (M ± 2 SE). The symbols represent the concentration in 10^7 nucleated cells.

3.3.3. Renal elimination (Fig. 3.11)

To determine the renal elimination of Adriamycin, rats were caged in metabolic cages designed by Van Bezooyen (personal communication), in which urine is collected without the chance of contamination by stools. Fig. 3.11 shows the cumulative renal elimination of Adriamycin in 72 h in normal animals. The mean volume of urine in which this amount of Adriamycin was excreted was 21 ml. The renal function as measured by the creatinine content of the serum changed significantly during progress of the disease and was actually diminished at day 15 (Fig. 3.12).

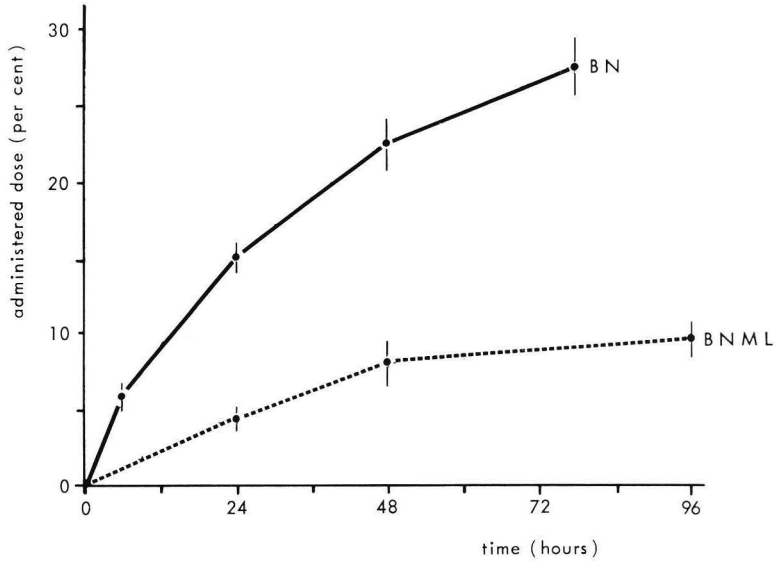


Figure 3.11

Cumulative urinary excretion of Adriamycin following a single intravenous bolus injection of 7.5 mg.kg^{-1} (M ± 2 SE) in normal rats.

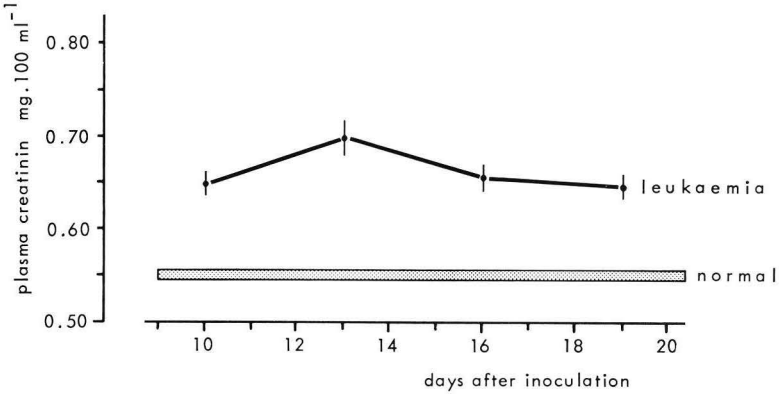


Figure 3.12

Plasma Creatinine content during development of the BNML (M ± 2 SE).

3.3.4. Biliary excretion (Fig. 3.13).

To determine the excretion of Adriamycin via the biliary route, 9 animals were anaesthesized with Ketamine hydrochloride and, using a microscope, a teflon drain with a diameter of 1 mm was inserted into the biliary duct. The drain was led through the skin and inserted into a 5 ml tube. During the sampling of the bile the rats were immobilized in a specially designed cage (Hagenbeek, 1977). Saline (0.9%) was administered intravenously at a volume of 2 ml per hour by means of an infusion pump during the sampling procedure in order to maintain a physiologic fluid balance. After the anaesthesia was finished, Adriamycin was administered intravenously in a single dose and the bile was collected at regular intervals. In Fig. 3.12, the cumulative biliary elimination during 24 h is plotted. A rapid initial excretion can be observed during 6 h, after which drug elimination decreases. These observations are from healthy rats, since 15-day leukaemic rats do not survive the surgical procedure.

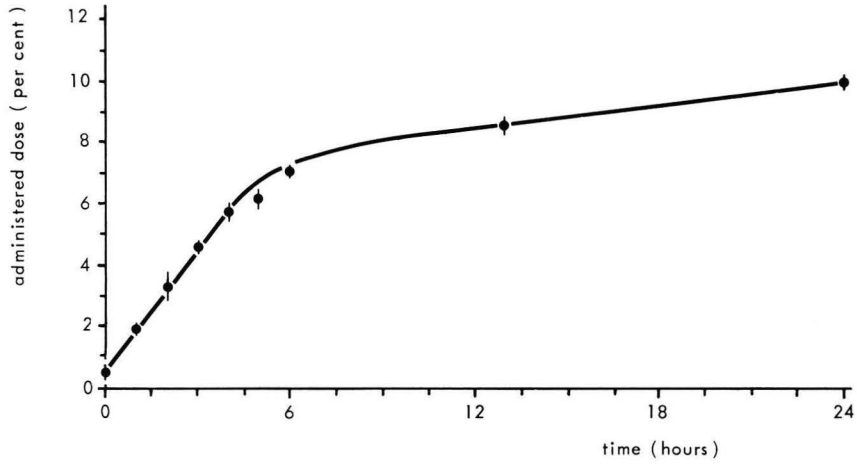


Figure 3.13

Biliary excretion of Adriamycin in normal rats following 7.5 mg.kg^{-1} (summation). The values represent the means of 9 animals (M ± SD).

3.3.5. Metabolism

Using the chromatographic procedure described in Chapter 3.2.2, in the first 24 h following administration of the drug, no metabolites

of Adriamycin could be demonstrated in plasma of normal and leukaemic rats up to 8 hours following drug administration. After this interval, 5% of the detectable fluorescence was produced by Adriamycinol. In urine, both Adriamycinol and Adriamycinone were present starting from 36 hours after injection of the drug.

3.4 DISCUSSION

The disappearance of Adriamycin in normal rats may be regarded as a triphasic process characterized by an initial rapid disappearance with an approximate duration of 25 min in the plasma followed by a second phase of redistribution and a terminal, quite long phase with a duration of more than 30 h. These findings are in agreement with data found in patients (Benjamin, 1975), which means that the rat is a good model for studying Adriamycin pharmacokinetics. The first phase in the plasma disappearance curve shows a linear pattern and represents a first order excretion phase to the various tissues. Conventional pharmacokinetic two-compartment systems, originally described by Wagner (1975), assuming an equal distribution of the drug in the plasma and in the well perfused organs are not sufficient to explain the triphasic pattern of the plasma disappearance curve. This is possible only if a multicompartment model in which various organs or tissues show different properties for the uptake and excretion of Adriamycin from the plasma is assumed. However, from the plasma curve only, it is not possible to ascertain which organs exhibit uptake or excretion properties other than the mean uptake which can be derived from the first phase of the plasma curve. Careful analysis of the distribution of the agent in several organs is therefore needed in order to discover where Adriamycin accumulates and how long it remains in each organ. Knowledge of this subject may reveal relevant information on the relation of a given plasma sample to the concentration in a given organ or tissue.

Analysis of the plasma disappearance curve of Adriamycin after 7.5 $mg.kg^{-1}$ intravenously reveals that, after 10 min, the concentration is 1.41 $\mu g.ml^{-1}$. Keeping in mind that a total of 1240 µg was injected into a rat of 165 g and the plasma volume amounts to 5.7 ml, the conclusion is that Adriamycin redistributes very rapidly to the tissues. Calculated per gram of tissue, a great part of the administered dose accumulates in bone marrow cells of both BNML and BN rats. However, determinations of the concentrations were performed only in

the femurs; therefore, a different uptake pattern in various parts of the bone marrow is theoretically possible. Assuming equal properties of bone marrow cells independent of their location in the body in respect to the uptake and retention of Adriamycin and supposing a total number of 4×10^9 bone marrow cells in the body, these data indicate that 25.8% of the total dose is accumulated in the normal bone marrow at 8 hrs. In BNML rats, the accumulated drug at this time amounts to 132 µg (10.6%), which is less than in normal rats, but still considerably more than the mean body concentration. In view of the severe toxicity of Adriamycin to the normal haemopoietic system, which is merely situated in the bone marrow, this means that this specific toxicity is not only the result of the fact that the haemopoietic system consists of rapidly dividing cells but also of the existence of high concentration during a prolonged period in this organ. Unfortunately, no literature data are available on the bone marrow concentration of Adriamycin *in vivo*, although Andersson et al. (1979) using whole body autoradiography with ^{3}H-Daunomycin have also found a considerable accumulation for a long period in mouse bone marrow. In leukaemic animals, the bone marrow concentration is considerably lower. Since the femoral cellularity has decreased in the leukaemic animal (Table 3.1), it can be concluded that leukaemic cells, which contribute to a great extent to the population of the femoral marrow, take up Adriamycin in smaller amounts than do normal haemopoietic cells. This might well be the result of cell specific characteristics with respect to drug uptake, as well of a reduced bioavailability of Adriamycin to femoral cells in the presence of leukaemia induced changes in organ structure. Additional information comes from *in vitro* uptake studies (Chapter 4).

A similar phenomenon can be observed in the spleen, where, during the observation period (24 h), the concentration of Adriamycin per g of spleen tissue is constantly high in both normal and 15-day leukaemic rats. However, the concentration in the leukaemic organ is significantly lower but shows the same disappearance pattern as in the normal organ. This may partly be the result of the different characteristics of BNML and normal spleen cells (mostly lymphocytes) regarding drug uptake. However, in view of the impressive increase in the spleen weight from 0.35 g to 1.28 g at day 15, reduced bioavailability of Adriamycin to the individual cells in the spleen as a result of pathological deviations from the normal vascular structure should be considered as a possible causative factor in the observed reduction of the intracellular concentration. This resembles that observed in bone marrow infiltrated with large numbers of leukaemic cells. Although the

total amount of Adriamycin present in a leukaemic spleen is higher than in the normal spleen, the concentration per cell is lower. At the same time, an increase in plasma concentration can be observed in the early 8 h. A redistribution of Adriamycin presumably occurs when a certain number of tumour cells is present in the body. Therefore, the relation between the plasma concentration and the concentration in organ infiltrated by tumour cells is different. Furthermore, increase in spleen weight and variations in the population of BN and BNML bone marrow cells occur during progression of the disease (Hagenbeek, 1977). At least in the spleen, therefore, an even more considerable deviation of the concentration from that in the normal spleen can be expected in the terminal stage of the disease. In that case, it is impossible to quantify the concentration of Adriamycin in the spleen from the plasma curve, since no information is available on the exact relation between these two organs.

The liver, which has increased in weight by a factor of 1.72, does not show significant deviations from the original concentration-time history. Other organs such as the lungs, the heart and the kidneys show the same disappearance curve in normal and leukaemic animals. On histological examination, no leukaemic infiltration into these organs can be demonstrated and no increase in weight is observed (Hagenbeek, 1977).

The quantitative differences in the plasma curve between BN and BNML rats can be attributed to the lower drug levels in leukaemia infiltrated organs, spleen and bone marrow. Table 3.5 summarizes the drug balance in normal and leukaemic animals.

From these data, the well-known cardiotoxicity of Adriamycin, which is also observed in this rat strain (Sonneveld, 1978), cannot be attributed to higher drug levels in the heart than in comparable organs such as the kidneys and the lungs. In the present study, the maximum concentration and the duration of cytotoxic levels in the heart were comparable with those in the lungs and even lower than those in the kidneys. Previously, Donelli had found cardiac levels of Adriamycin to be comparable with those in lungs of normal mice and pointed out that drug induced cardiotoxicity does not depend on intracellular drug levels (Donelli et al., 1977).

In these animals, the excretion of Adriamycin occurs according to the pathways which have been described (Benjamin et al., 1973; 1974; Bachur, 1975). However, only the amount of tritiated Adriamycin in the faeces was determined by these investigators, while in the present study, the concentration in the bile was investigated. Since the contribution of an enterohepatic recirculation is not defined, it is un-

Table 3.5

ADRIAMYCIN MASS BALANCE IN BN AND BNML* RATS FOLLOWING 7.5 $mg.kg^{-1}$ i.v.
(Adriamycin per cent of administered dose)

	BN			BNML		
Time intervals (h)	1	4	8	1	4	8
spleen bone marrow liver	18.9	32.8	29.0	22.7	20.9	19.3
heart lungs kidneys plasma	4.6	3.8	2.6	4.7	3.2	3.3
urine bile	0.4	6.6	17.5	0.5	7.6	16.4
other	76.1	57.2	50.9	72.1	68.3	60.8

*At day 15 after inoculation of 10^7 leukaemic spleen cells.

certain as to how far the results can be compared. An 8% biliary elimination in 24 h, compared with a 50% excretion in the faeces in 7 days, does not provide strong evidence for the presence of such an enterohepatic circle. The urinary route is the major pathway of excretion in the normal rat (26% in 72 h). However, the decrease in normal renal elimination function, as measured by the content of creatinine in the peripheral blood, results in a decreased excretion of Adriamycin in leukaemic rats. This decrease of elimination leads to prolonged elevated levels of Adriamycin in leukaemic rats. A great proportion of the effective tumour reducing function and the toxicity to normal tissues is probably a direct result of this phenomenon, especially since the elimination of Adriamycin from many organs is slow. Besides the increase in tissue levels due to disturbances in elimination, differences in uptake by normal haemopoietic and leukaemic cells may also be of influence. Although the antitumour activity of Adriamycin is not directly correlated with the intracellular concentration within one cell line (Carter, 1975), differences in drug uptake by different cell lines, e.g., leukaemic and normal haemopoietic cells, may account for dissimilar sensitivities. These mechanisms will be further discussed in Chapter 4.

The results discussed in this Chapter indicate that there are significant differences between normal animals and animals with a leukaemic cell load with respect to the general distribution of Adriamycin.

These differences cannot be quantitated solely from the plasma curve. In both normal and leukaemic animals, various organs show selective accumulation of the drug. Therefore, it can be concluded that an estimation of the drug level at the tumour site cannot be performed without information on both the tumour load and the normal distribution of the drug. Since conventional pharmacokinetic models are insufficient to perform such an estimate, a new approach has been developed to solve the problem of unknown distribution parameters and will be described in Chapter 5.

CHAPTER 4

BIOLOGICAL ACTIVITY OF ADRIAMYCIN IN VITRO

4.1 INTRODUCTION

During the last few years, the action of Adriamycin has been studied in many different cell systems in vitro as well as in vivo, mainly in comparison with the action of the related drug Daunomycin. It became apparent that Adriamycin is closely related to Daunomycin not only in its structure, but also in its effect at the cellular level. Both agents belong to the same group of antibiotics and are supposed to exert their cytotoxic effects during the S and G_2 phases of the cell cycle.

The majority of the in vitro studies on Adriamycin activity concerns its inhibitory effect on the DNA and RNA synthesis of cultured Hela or L1210 cells (Silvestrini et al., 1970; Lutz and Stacher, 1975; Wantzin and Killmann, 1977). Presumably due to the binding of the drug to nucleic acids, it inhibits both DNA and RNA synthesis and the viral, bacterial and mammalian cell DNA-dependent DNA polymerase and bacterial RNA polymerase (Wang et al., 1972; Zunino et al., 1974; 1975). A clear relationship between the binding of the drug to nuclear structures and the inhibitory effect was demonstrated by Momparler and co-workers, who found that Adriamycin exhibited a greater inhibition of DNA-dependent DNA polymerase than of RNA polymerase in isolated nuclei and in cell free enzyme extracts (Momparler et al., 1976). However, in intact A(T1)Cl-3 hamster fibrosarcoma cells, Adriamycin inhibited both DNA and RNA synthesis to the same extent. These differences may be attributed to either preferential binding of the drug to chromosomal DNA in the cell or to metabolic transformation of the drug.

A certain difference between Adriamycin and Daunomycin has been observed with respect to the inhibitory effect on DNA and RNA synthesis. At equal doses exhibiting comparable antimitotic effects, the DNA synthesis rate was inhibited more by Daunomycin than by Adriamycin (Silvestrini, 1970). However, here the sensitivity of the cell line probably plays a major role, as can be concluded from various contradictory reports. The in vitro studies of Wang et al. (1972) (using L1210 cells) and of Kim and Kim (1972) (using Hela cells) indicate

ing (Nuclear Chicago, Mark II counter). Labelled cells were harvested on glass fibre filters (type A-E, Gelman, Ann Arbor, Mich., USA). The air dried filters were placed in scintillation vials (Packard Instr., Zurich, Switzerland) and 2 ml of a toluene-based scintillation fluid (50 mg POPOP and 4 g PPO per liter toluene) were added.

4.2.3. DNA and RNA synthesis in spleen and bone marrow cells

Normal and leukaemic animals were anaesthesized with ether and killed by cervical dislocation; spleens were removed, weighed and collected in Hanks'. The femurs were removed, cut and also placed in Hanks'. Spleens were separately finely minced with a pair of scissors and filtered repeatedly through six layers of nylon gauze. The bone marrow cells were collected by repeatedly flushing the femoral shaft with Hanks' solution using a bent needle placed on a syringe. The collected bone marrow was filtered through six layers of nylon gauze. The cell suspensions were collected in Falcon tubes and suspended with Hanks' to a final concentration of 10^7 cells.ml^{-1}. Adriamycin dissolved in saline was added at different concentrations in a final volume of 0.1 ml and the cells were incubated in a waterbath at 37^{o} C in closed tubes for one hour. Then, ^{3}H-TdR at a dose of 1 µC.10^{-7} cells or ^{3}H-UdR at a dose of 1.5 µC.10^{-7} cells was added in a final volume of 0.1 ml; the tubes were shaken and incubated again for 30 minutes. After this period, the incorporation was terminated by adding an ice-cold solution of unlabelled thymidine in saline. After repeatedly washing the cells with saline, they were harvested on glass fibre filters. After drying overnight at 37^{o} C, the filters were placed in scintillation vials and scintillation fluid was added. Results were expressed as the absolute number of counts per min (cpm) of triplicate samples as a function of the concentration of Adriamycin.

4.2.4. Cellular influx and efflux of Adriamycin

Animals were anaesthesized with aether and killed by cervical dislocation. Bone marrow cells from the right femur were obtained as described in section 4.2.3. The cells were suspended in Hanks' in a volume of 10^8 per ml. Adriamycin was added at various concentrations in a final volume of 0.1 ml and the cells were incubated at 37^{o} C for one hour in a water bath. After the incubation period, the cells were washed two times and either placed at 4^{o}C or reincubated in Adriamy-

cin-free medium. These reincubated cells were washed afterwards with saline and placed on ice. The intracellular content of Adriamycin was determined spectrophotometrically. After drug extraction from the cells according the method described in section 3.2.5, the relative fluorescence, as obtained by comparison with known standards, was used to determine the intracellular drug concentrations.

4.2.5. Pulsecytophotometry

The cytotoxic action of Adriamycin in vitro was assessed as a function of the drug concentration, using the incubation procedure described in section 4.2.2. However, instead of ^{3}H-TdR, cold thymidine (20 $\mu g.10^{-7}$ cells) was added. To investigate the cell cycle phase-specific action of Adriamycin as a function of its concentration in the incubation medium, two separate experiments were performed.
A) For studying the kinetic status of only cells killed during the incubation period, all incubated cells were washed twice in Hanks' solution by centrifugation at 200 g and the pellet was resuspended to a final concentration of about 1 x 10^{7} cells.ml^{-1} in a solution of 5 per cent of the fluorescent dye Propidium Iodide (PI) (CalBiochem, La Jolla, Ca. 92037, USA) in saline (Krishan, 1975). Since PI enters dead cells only, the ratio of dead/viable cells and the DNA distribution of dead cells could be determined by measuring the relative number of cells exhibiting PI related fluorescence at 590 nm using a FACS II cell sorter (Becton and Dickinson Company, Mountain View, USA), which was equipped for this purpose with an argon ion laser (Spectra Physics 164-05, 1 Watt). Here, the excitation wavelength is 514.5 nm, while the emission of PI-stained cells is determined by a S-20 type photomultiplier with filters: Ditric 6200 cut on plus dichroic beam splitter (570 nm) (Melles Griot, Arnhem, The Netherlands). B) Additionally, DNA histograms were prepared of all incubated cells simultaneously, either or not killed by Adriamycin. For this purpose, cells were washed in Hanks' solution by centrifugation at 200 g and the pellet was resuspended to a final concentration of 10^{7} cells.ml^{-1} in a hypotonic solution containing 0.1% sodium citrate and 5% PI. With this procedure cells disrupt in the hypotonic solution and the DNA distribution of PI stained cell nuclei can be determined within 24 hours.

4.2.6. Assay for the quantification of leukaemic and haemopoietic clonogenic cells: leukaemic colony forming unit-spleen (LCFU-S) and colony forming unit-spleen (CFU-S)

Low numbers of BNML cells (5×10^3 to 5×10^4) injected intravenously into nonirradiated recipient BN rats grow out into colonies which can be counted on the surface of the spleen at day 19 after injection. A linear relationship has been found between the number of injected cells and the number of spleen colonies (Van Bekkum et al., 1976). On microscopic inspection, these colonies consist of leukaemic blast cells. The leukaemic origin of the colonies has been confirmed by injecting cells obtained from these nodules into secondary recipients. When too many clonogenic cells are inoculated, the colonies are confluent at day 19 and cannot be counted. In the present study, BNML bone marrow cells treated with Adriamycin at various concentrations for 1 h *in vitro*, were injected into BN recipients at concentrations varying from 10^3 to 10^6 per recipient and the number of spleen colonies was counted at day 19.

The number of pluripotent haemopoietic stem cells (HSC) in the BN rat can be determined with the CFU-S assay originally described by Till and McCulloch (1961) and modified by Comas and Byrd (1967) and Van Bekkum et al. (1976). With this technique, bone marrow cells are injected intravenously into lethally irradiated mice. The stem cells which are trapped in the spleen give rise to colonies which can be counted macroscopically at day 9 after injection. Those cells which give rise to a colony are defined as colony forming units-spleen (CFU-S). A linear relationship is found between the number of injected bone marrow cells and the number of spleen colonies in two recipient species: the BN rat and F1 hybrids of CBA x C57BL mouse strains.

In this study, normal bone marrow cells were incubated *in vitro* with various concentrations of Adriamycin for 1 hour, and injected into F1 hybrid mice of C57BL/LiRij x C3H/LwRij. The recipients had received 10.25 Gy gamma total body irradiation before injection. Nine days after injection of the cell suspension, colonies on the surface of the mouse spleen were counted after fixation of the spleen in Tellyesniczky's solution (ethanol 70%, formaldehyde 36%, acetic acid 100%: 20:1:1). The aim of this study was to investigate whether these clonogenic assays produce comparable results to those from the DNA and RNA inhibition assays, which, in fact, measure loss of cell function rather than loss of cell clonogenecity.

4.3 RESULTS

4.3.1 Adriamycin and cell lethality

When the effect of Adriamycin on various parameters of cell viability is determined, some quantitative differences can be observed. For example, the inhibition of DNA synthesis (as measured by inhibition of ^{3}H-TdR uptake) as a function of the concentration of Adriamycin in the incubation medium is significantly influenced by the duration of the incubation period. In these experiments, leukaemic bone marrow cells were incubated with various concentrations of Adriamycin for periods from 30 min to 48 h at 37^{o} C. Thereafter, cells were washed, ^{3}H-TdR was added and were further treated as described in section 4.2.3. During the incubation, the stability of the osmolarity and the pH of the suspension was monitored using an Advanced Osmometer (Massachusetts, USA) and a PHM 61 Radiometer (Copenhagen, Denmark), respectively. It appeared that no significant variations in osmolarity or pH occurred during incubation periods of up to 2 h. As shown in Fig. 4.1 the uptake of ^{3}H-TdR in the suspended cells is inversely related to the duration of the incubation period.

After periods of incubation longer than 2 h hardly any effect of Adriamycin on the uptake of ^{3}H-TdR can be demonstrated, while shorter periods lead to a concentration dependent inhibition of the ^{3}H-TdR uptake. However, even at 48 h, a still measurable uptake is observed, which should probably be attributed to cells which are relatively insensitive to the action of Adriamycin such as lymphocytes and macrophages. It can be concluded that incubation periods of 30 min to 1 h provide the optimal conditions for measuring the Adriamycin induced inhibition of DNA synthesis. However, if a 30 min incubation period is used low concentrations of Adriamycin (0.01 - 1.0 µg.10^{-7} cells) prove to be insufficient for adequate inhibition, and even induce acceleration of DNA synthesis (Fig. 4.2). For incubation periods of 1 h, a clear concentration dependent inhibition is found; therefore in further experiments this period was used (Fig. 4.3).

In Table 4.1, the Adriamycin induced inhibition of DNA synthesis in leukaemic cells is compared with its effect on the viability of BNML cells and their clonogenecity. For viability measurements the eosin staining method was used, while the LCFU-S assay was employed to determine the reduction of clonogenic leukaemia cells after Adriamycin treatment. The reduction of clonogenic leukaemia cells compares well with the inhibition of DNA synthesis at drug concentrations 10 µg. 10^{-7} cells, while the number of dead cells as determined with eosin

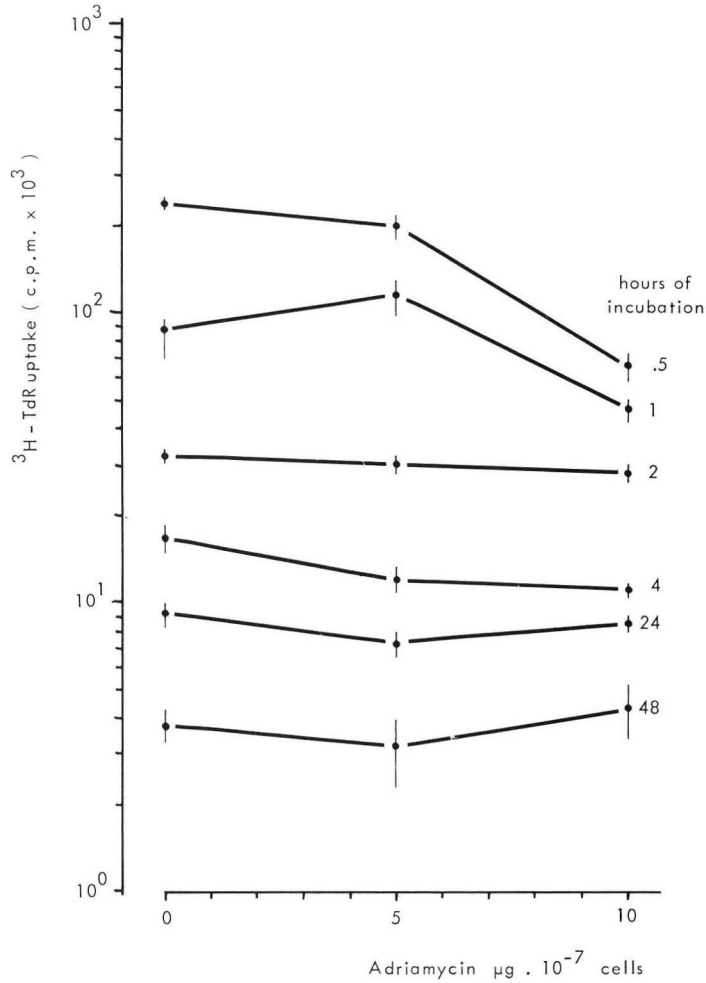


Figure 4.1:

Incorporation of ^{3}H-TdR in leukaemic bone marrow cells after various periods of incubation with different concentrations of Adriamycin. Results are expressed as radioactivity present in the cells and represent the M ± 2 SE of 5 experiments.

uptake is not altered by Adriamycin. This finding implicates that data about DNA synthesis apparently underestimate the Adriamycin induced kill of clonogenic leukaemic cells. This is probably because of the fact that the LCFU-S assay reflects only clonogenic leukaemic cell kill, while the ^{3}H-TdR uptake of all cells is measured, including those cells, either leukaemic or not, which do not synthesize DNA.

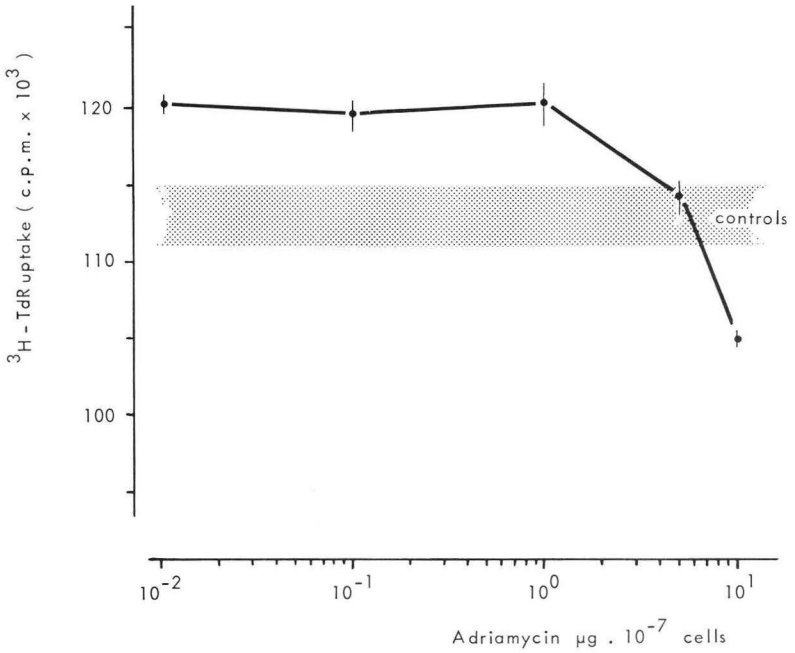


Figure 4.2:

Incorporation of ^{3}H-TdR in leukaemic bone marrow cells after 30' of incubation with Adriamycin (M ± 2 SE of 5 experiments).

Therefore, the measurement of DNA synthesis may well be used to observe the effect of Adriamycin on BNML cells. In Table 4.2 the reduction of clonogenic leukaemic cells and clonogenic haemopoietic cells as a function of the concentration Adriamycin in the incubation medium is compared. It appears that the reduction of CFU-S at the two lower concentrations of Adriamycin is less than that of LCFU-S. This difference is reflected in the inhibition of DNA synthesis of leukaemic and normal bone marrow cells, as will be pointed out in 4.3.3.

4.3.2 Influx and efflux of Adriamycin

Incubation of BN and BNML femoral bone marrow cells for 60 min in the presence of different concentrations of Adriamycin leads to an intracellular accumulation of the drug which is dependent on its concentration in the medium. As shown in Table 4.3, isolated BNML cells take up significantly more Adriamycin than do BN cells at drug concen-

TABLE 4.3

UPTAKE AND INTRACELLULAR RETENTION OF ADRIAMYCIN IN BN AND BNML CELLS*

Extracellular concentration of Adriamycin $\mu g.10^{-7}$ cells	BN		BNML	
	Uptake	Retention	Uptake	Retention
0	0	0	0	0
1	0.4	0.2	0.8	0.5
10	3.2	2.7	5.0	4.4
50	7.7	6.1	9.5	8.8
100	9.4	8.3	13.2	12.0

*Expressed as $\mu g.10^{-7}$ cells.

trations of 10 µg or more per 10^7 cells ($p < 0.001$). The two cell types do not differ in their capacity to retain the intracellular amount of drug when placed in a drug-free incubation medium. The remaining fluorescence (which is a measure for the intracellular amount of Adriamycin) in leukaemic cells exceeds that in normal bone marrow.

4.3.3 Adriamycin and the DNA synthesis

It has been reported several times that the incorporation of thymidine into cells is paralleled both in magnitude and in time by the DNA polymerase activity (Loeb and Agarwal, 1971; Munch-Petersen et al., 1973; Tyrsted et al., 1973). Since especially the inhibition of DNA polymerase is a major manifestation of the cytotoxic action of Adriamycin, the inhibition of tritium labelled thymidine incorporation as a function of the concentration of Adriamycin present was employed to investigate the dose-effect relation between Adriamycin and both normal haemopoietic and leukaemic cells *in vitro*. When bone marrow cells of BN or BNML origin are incubated in the presence of various concentrations of Adriamycin and ^{3}H-TdR subsequently is added, the uptake of the latter compound is inhibited to a different extent in the two cell types. Low concentrations of Adriamycin (0.01-1.0 µg. 10^{-7} cells) lead to a decreased uptake of ^{3}H-TdR in BNML cells, while a high concentration (100 µg.10^{-7} cells) inhibits the uptake of ^{3}H-TdR

to 67% in BN and to 35% in BNML cells (Fig. 4.3). It is clear from the figure that the control numerical values for the uptake of ^{3}H-TdR in BN cells are twice those of BNML bone marrow cells. This could be due to a relatively higher percentage of BN cells being in the DNA synthesizing phase of the cell cycle (S phase). According to Hagenbeek (1977) the labelling index (representing the percentage of cells in the S phase) is 58% for normal rat myeloblasts and 30-43% for BNML cells, which findings may account for the observed difference in ^{3}H-TdR uptake in control samples. In section 4.3.5 this discrepancy will be further evaluated. From the effect of Adriamycin on ^{3}H-TdR uptake (which in fact is a measure for the DNA synthesis), it may be concluded that a difference is demonstrable with respect to the sensitivity of BN and BNML bone marrow cells. In suspended normal spleen cells the uptake of ^{3}H-TdR of control samples is significantly less ($p < 0.001$) than that of BN bone marrow cells; this could be expected, since a normal spleen cell population is mainly composed of nonproliferating lymphocytes. Addition of various concentrations of Adriamycin does not significantly alter the uptake of ^{3}H-TdR (Fig. 4.4). In

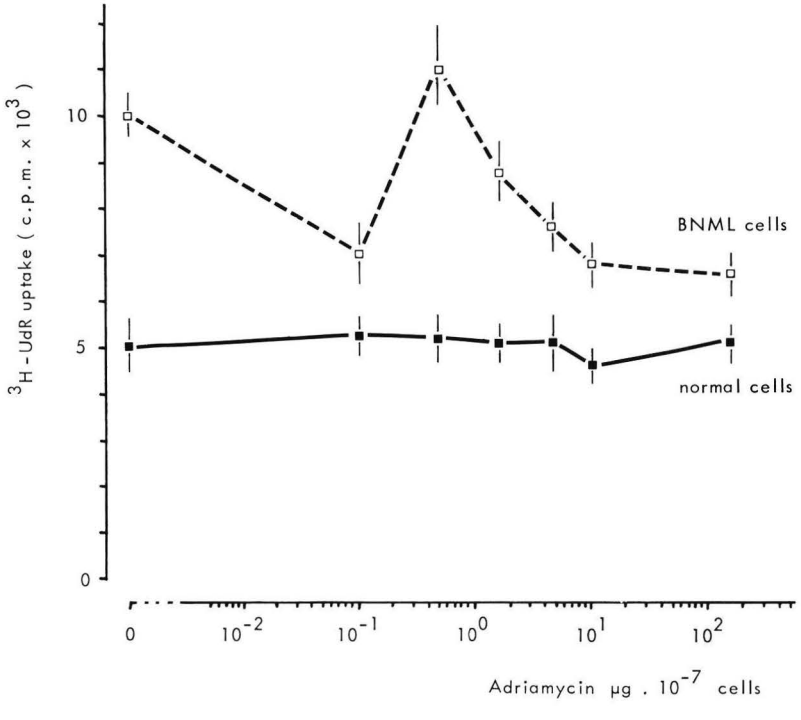


Figure 4.4:

Incorporation of ^{3}H-TdR in BN and BNML spleen cells following 1 h of incubation with Adriamycin in vitro (M ± 2 SE of 5 experiments).

BNML spleen cells, low concentrations of Adriamycin (0.01-0.1 $\mu g.ml^{-1}$) inhibit the uptake of ^{3}H-TdR to 70% without any further decrease at higher drug concentrations. This inhibition is less than in BNML bone marrow cells, which may be explained by the fact that, in BNML animals, the extramedullary haemopoiesis takes place mainly in the spleen which implies that a suspension of spleen cells from these animals is composed of both haemopoietic and leukaemic cells. Furthermore, the labelling index of BNML spleen cells is higher than that of BNML bone marrow cells (0.48 versus 0.36). This might indicate that, because of the high percentage of S-phase cells, higher concentrations of Adriamycin are required to further inhibit the DNA synthesis. A strange but very reproducible observation in BNML spleen cells is the increase in ^{3}H-TdR uptake at the 5.0 $\mu g.ml^{-1}$ level, for which no explanation is available at present.

Although the cytotoxic effect of Adriamycin has not been reported to be exerted exclusively during one cell cycle phase, its activity is more pronounced when used in cells which have been previously synchronized with Arabinoside-Cytosin (ara-C) (Colly, 1980). It is known that an appropriate dose of ara-C *in vivo* leads to to recruitment of non-proliferating BNML cells into cycle with an increased (up to 60%) number of cells in the DNA synthesis phase of the cell cycle at 12 h following injection of ara-C (Aglietta and Sonneveld, 1978; Aglietta and Colly, 1979). This phenomenon was used to investigate the action of Adriamycin *in vitro* on synchronized cells. For this purpose bone marrow and spleen cells were obtained from normal and BNML rats which had been injected with ara-C (200 $mg.kg^{-1}$ i.v.) 12 h earlier. Fig. 4.5 shows the *in vitro* effect of Adriamycin on BNML and BN bone marrow and spleen cells treated with ara-C *in vivo*. Of the population of BNML cells at the time of incubation $\pm$ 60% is in the S phase of the cell cycle (Aglietta and Sonneveld, 1978). Addition of Adriamycin leads to a concentration dependent reduction in ^{3}H-TdR uptake in these cells down to 10% of the control values at the highest concentration of Adriamycin (10 ug. 10^{-7} cells). The same observation is made in suspended femoral BN cells. In all of these cell types (femoral BN + BNML, splenic BNML) the control ^{3}H-TdR uptake is twofold the value obtained with comparable cells, not pretreated with ara-C. However, in BN spleen cells which are mainly nondividing lymphocytes, no alterations are observed when compared with untreated splenic cells.

Apparently, pretreatment with ara-C increases the sensitivity of nonexponentially proliferating cell populations to the action of Adriamycin, due to the greater number of cells in the S phase of the cell cycle. However, the observation that, after pretreatment with

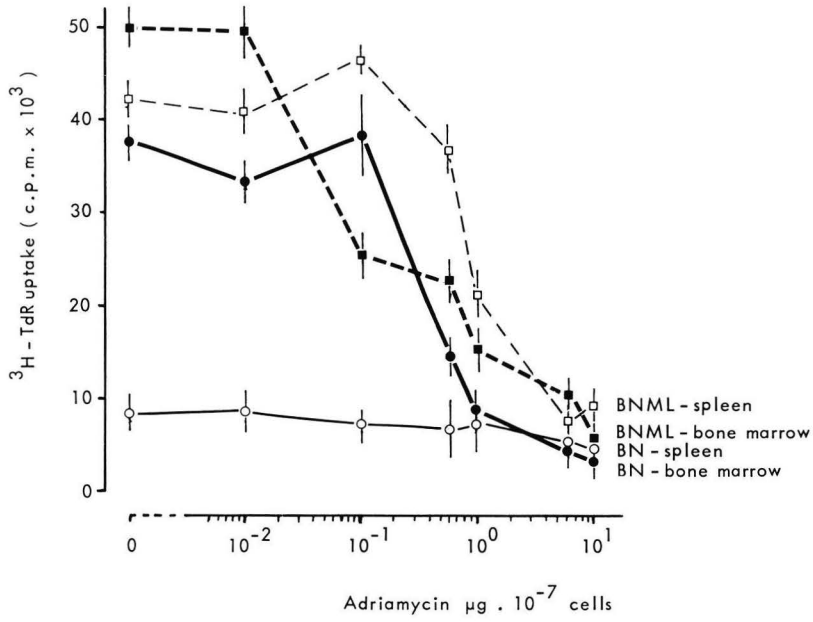


Figure 4.5:

Incorporation of ^{3}H-TdR in BN and BNML femoral bone marrow and spleen cells pretreated with ara-C *in vivo* following 1 h of incubation with Adriamycin *in vitro* (M ± 2 SE of 3 experiments).

ara-C, Adriamycin reduces the ^{3}H-TdR uptake to lower values than without pretreatment, requires another explanation. Ara-C blocks proliferating cells at the G_1-S boundary of the cell cycle and simultaneously triggers nonproliferating cells into cycle (Aglietta and Colly, 1979). At the moment that the blockade is terminated, a synchronous wave of cells enters the S phase, which occurs at 12 h following administration of ara-C. The early S phase, however, is the predominant site of action of Adriamycin (see section 4.3.5). Consequently, an almost total inhibition of DNA-synthesizing activity of the synchronized cells can be expected. This situation thus, is totally different from that of a cell population which is randomly distributed over the S phase, where only early S phase cells are inhibited with respect to ^{3}H-TdR uptake; late S phase cells still may take up ^{3}H-TdR, irrespective of the presence of Adriamycin.

4.3.4 Adriamycin and RNA synthesis

The effect of Adriamycin on RNA synthesis as reflected by its effect on the uptake of ^{3}H-Uridine (^{3}H-UdR) into BN and BNML femoral bone marrow cells is presented in Fig. 4.6. As with DNA synthesis a concentration dependent reduction in the ^{3}H-UdR uptake is observed, which is more pronounced in BNML cells than in BN cells. It is remarkable that the uptake of ^{3}H-UdR in leukaemic cells is completely inhibited at the highest concentration of Adriamycin (50 $\mu g.ml^{-1}$), which confirms that RNA polymerase is blocked by Adriamycin to greater ex-

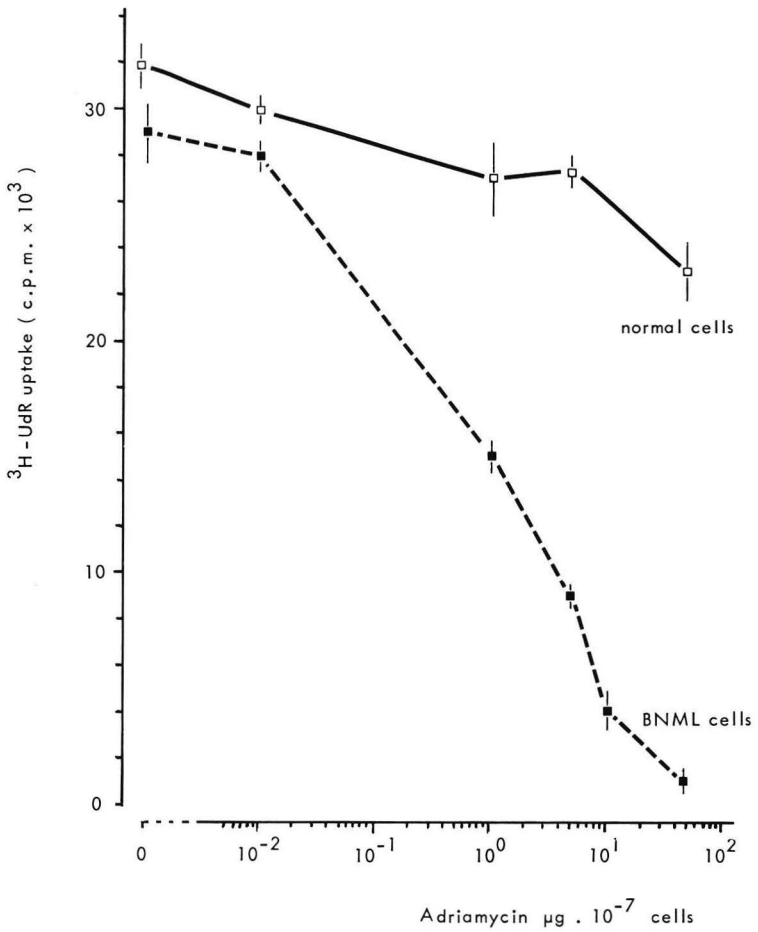


Figure 4.6:

Incorporation of ^{3}H-UdR in BN and BNML femoral bone marrow cells following 1 h of incubation with Adriamycin *in vitro* (M ± 2 SE of 5 experiments).

tent in these cells. However, inhibition of RNA synthesis apparently does not represent an essential mechanism of Adriamycin induced cytotoxicity, in view of the differences in inhibition of DNA synthesis and reduction in LCFU-s. The inhibitory effect of Adriamycin on suspended splenic BNML cells is less impressive, although comparable with its effect on ^{3}H-TdR uptake in these cells (Fig. 4.7). Normal spleen cells are not sensitive to Adriamycin in this respect.

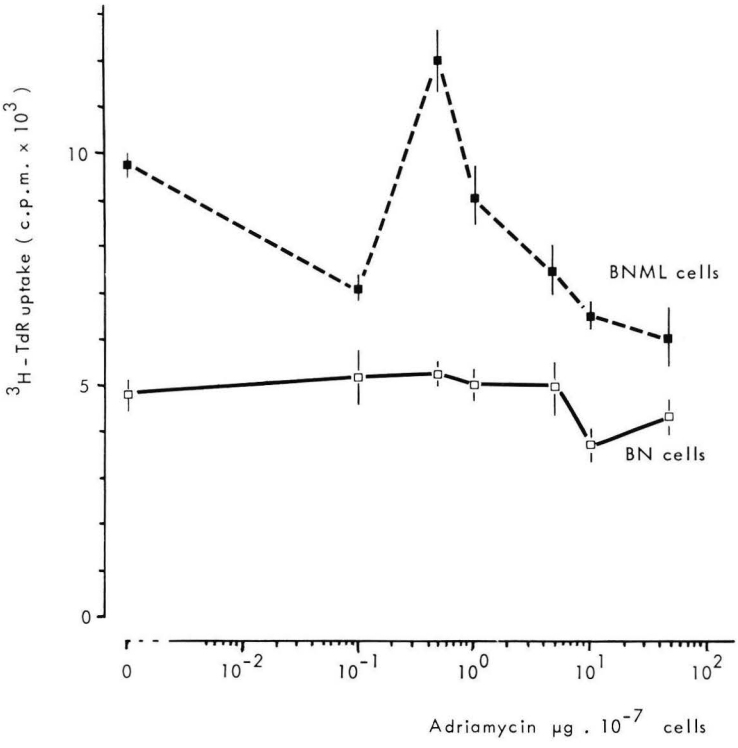


Figure 4.7:

Incorporation of ^{3}H-UdR in BN and BNML spleen cells following 1 h of incubation with Adriamycin *in vitro* (M ± 2 SE of 5 experiments).

4.3.5 Adriamycin and the cell cycle

As mentioned in Chapter 2 and the introduction to this Chapter, there is confusion concerning the phase of the cell cycle, in which Adriamycin primarily exerts its cytotoxic action. The conflicting data

cell cycle. A low concentration (1 $\mu g.10^{-7}$ cells) of Adriamycin does not alter this pattern (Fig.4.10.c).However, 10 $\mu g.10^{-7}$ cells leads to a preferential accumulation of dead cells in the G_1 and the early S phase of the cell cycle (Fig. 4.10.d). In BNML bone marrow cells essentially similar observations are made in untreated cells (Fig. 4.11a and b), while here also at the lowest concentration Adriamycin kills cells in the G_1 and early S phase (Fig. 4.11.c and d).

These observations indicate that Adriamycin kills both BN and BNML bone marrow cells primarily in the G_1 and early S phases of the cell cycle and that BNML cells are more sensitive than are BN cells.

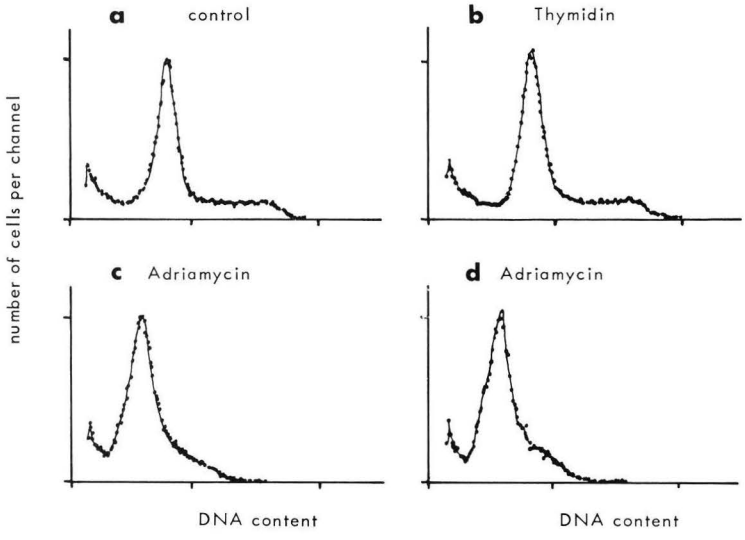


Figure 4.11:

DNA histograms of BNML bone marrow cells killed during 1 h of incubation. Treatment: a) NaCl 0.9%; b) thymidin 20 $\mu g.10^{-7}$ cells; c) Adriamycin 1 $\mu g.10^{-7}$ cells; d) Adriamycin 10 $\mu g.10^{-7}$ cells.

4.4 DISCUSSION

It has been suggested by Roger and Drewinko (1976) that only the reduction in clonogenic cells *in vitro* gives a reliable indication of the response to chemotherapy *in vivo*. Although this statement may certainly be valid for those cells which can be cultured *in vitro*, it leads to no valuable predictive assay which may be of help to quantify

antitumour effects of cytostatic drugs in vitro in those cells which cannot be cultured, such as in this BNML leukaemia and most human acute leukaemias. Therefore, in the present study, the relatively less valid assay for DNA synthesis inhibition has been compared with clonogenic assays for leukaemic and haemopoietic cells, which were previously shown to yield reliable data on the reduction in blast cells following chemotherapy. With this rat leukaemia, LCFU-s data can be well compared with data concerning the inhibition of DNA synthesis but not with data from the eosin staining method which prove to be inaccurate for monitoring cytotoxic drug effects. Furthermore, LCFU-S and CFU-S data agree with data concerning the inhibition of DNA synthesis of leukaemic and normal bone marrow cells, respectively. One restriction in the interpretation of data from the DNA synthesis is the fact that, at high concentrations of Adriamycin ($\geqslant$ 10 $\mu g.10^{-7}$ cells), the inhibition of ^{3}H-TdR uptake is not complete while the reduction in LCFU-S and CFU-S is. BNML and BN cells at these drug concentrations may synthesize DNA, but are no longer able to divide.

In the lower concentration range, however, data on the inhibition of DNA synthesis may be considered to represent the actual drug induced cell kill. Using this assay as an indicator for the effects of Adriamycin in vitro, several interesting differences can be observed between the sensitivity of BNML and normal bone marrow cells. Both BNML cells from both spleen and bone marrow exhibit Adriamycin induced inhibition of DNA synthesis at concentrations of 0.01 - 1.0 $ug.10^{-7}$ cells, while normal haemopoietic cells are not sensitive. This implies that in this concentration range Adriamycin is not myelotoxic but exerts significant cytotoxicity on leukaemic cells. Higher concentrations lead to cell kill of both BNML and haemopoietic cells. These killed cells are in the S phase of the cell cycle at the time of drug induced death, as can be concluded from analysis of DNA histograms, which show an accumulation of dead cells in the S phase. On the contrary, cells which are not killed by Adriamycin are normally distributed on the DNA histogram. Pulse cytophotometric analysis of BNML spleen and bone marrow cells incubated with Adriamycin shows an accumulation of dead cells in S phase at concentrations of 1.0 and 10 $\mu g.10^{-7}$ cells, while normal bone marrow cells show such an accumulation only at 10 $\mu g.10^{-7}$ cells. Obviously, the same drug concentration dependent difference in sensitivity as found with the inhibition of DNA synthesis can be detected between normal and leukaemic cells with this procedure. The observed differences might be attributed to specific properties of BNML cells and normal bone marrow cells with respect to the uptake of Adriamycin into the cell. With both cell types in-

creasing extracellular drug concentrations lead to an increasing intracellular drug content, which is, however, more pronounced in BNML cells. The two cell types do not differ in their properties of retaining intracellular Adriamycin when placed in a drug-free incubation medium.

It may be concluded from these data that mainly S phase cells are sensitive to the action of Adriamycin. Previous experiments by other investigators using human lymphoma cells had demonstrated a selective accumulation in the G_2 phase of the cell cycle after treatment with Adriamycin *in vitro* (Barlogie et al., 1976; Krishan et al., 1975). Apparently, the BNML cell line exhibits a different sensitivity to the cytokinetic action of Adriamycin; this had already been concluded by Colly (1980) and may be the result of the proliferation characteristics of BNML cells, which exhibit a long S phase duration. Pretreatment with ara-C increases the Adriamycin induced death of BNML and normal bone marrow cells. Still the S phase sensitivity is only valid in the presence of low concentrations of Adriamycin, since, according the LCFU-S and CFU-S data, high concentrations ($\geqslant 10$ µg.10^{-7} cells) kill all clonogenic cells and consequently also act on cells in the G_0 and G_2 phases. Therefore, the drug induced cell kill at high concentrations cannot be accurately determined with the ^{3}H-TdR uptake assay.

When these *in vitro* observations are translated to the *in vivo* treatment of acute myelocytic leukaemia, it may be cautiously concluded that the establishment of low tissue concentrations (0.1 - 1.0 µg.10^{-7} cells) in bone marrow and spleen will result in Adriamycin induced kill of leukaemic cells without severe harmful effects to normal bone marrow cells. However, *in vivo* concentrations of Adriamycin in bone marrow of BNML rats, which are obtained following a dose of Adriamycin which is presently employed in most AML clinical protocols, are in a range which produces death of both leukaemic and haemopoietic cells. Probably, the *in vivo* bone marrow concentrations of Adriamycin can be lowered by modification of drug dose. Therefore, the present results may indicate that the clinically observed haematological toxicity of Adriamycin can be reduced by employment of lower dosages without reduction in the antileukaemic activity of the drug.

CHAPTER 5

A MULTICOMPARTMENT MATHEMATICAL MODEL FOR THE DISTRIBUTION OF ADRIAMYCIN IN VIVO

5.1 INTRODUCTION

The development of chemotherapy into a valuable branch in the framework of cancer treatment has introduced the serious problem of extreme toxicity to the host, both general and specific. The harmful effects of intensive chemotherapy to rapidly dividing tissues are limiting factors in achieving the desired therapeutic result and occur to an extent which is generally unpredictable in the individual patient. Since the introduction of drug monitoring techniques, the assumption has been made that toxicity can be correlated with and eventually predicted from the plasma concentration (Wagner, 1975). Later experience showed that, for a number of cytotoxic agents, the plasma concentration poorly reflects drug concentrations in either target tissues or organs (Chen and Gross, 1979). Therefore, a more extended description of the drug kinetics, which should also regard the distribution characteristics in several tissues is required. Pharmacokinetic models represent a rational approach to this problem. As pointed out by Bisschoff (1975), these models should be based on quantitative predictive aims, e.g., they should be able to predict unknown concentrations in inaccessible tissues from known concentrations in plasma and other easily approachable fluids. Recent advances have been made with this approach for a number of cytotoxic agents (Harris and Gross, 1975; Chan et al., 1978; Dedrick et al., 1972, 1973a, 1978; Morrison et al., 1975; Bisschof et al., 1970, 1971; Dedrick et al., 1973b, 1978). As discussed by Chan and Gross (1979), these pharmacokinetic models are based on several assumptions: 1) flow limitation, which means that the tissue drug concentration is proportional to the arterial and venous drug levels; 2) the concentration in each subdivision of the model is homogeneous; 3) drug protein binding is linear in plasma and tissues. Correlations between plasma and tissue concentrations following different schedules of administration are investigated primarily in animal systems. Later application in man can be done by simple translation of the blood flow and organ volume parameters. How-

ever, there is extensive variation in the accuracy of these models, due to the number of subdivisions included.

One, two and multicompartimental models can be identified. In the one compartment open model (Fig. 5.1), the body is represented by a single compartment with volume V, assuming a homogeneous disposition at any moment during the observation period. There is no distribution phase and excretion from the body is assumed to be first order. In view of the unrealistic representation of the physiological situation (where most drugs are not equally distributed over the body), this model is not attractive for investigation of the disposition of anti-cancer drugs.

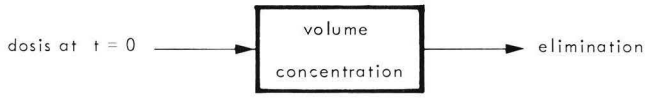


Figure 5.1:

The one-compartment open model with rapid bolus intravenous injection.

The two compartment open model assumes the existence of a concentration gradient between two separate regions of the body, generally the well and the poorly perfused tissues (central and peripheral compartment) (Teorell, 1937). Elimination from the system can occur from either compartment or both (Fig. 5.2). The rate of drug transport is a function of the drug concentration in both compartments (first order linear transport). If this postulate is not entirely valid, for example in the case of active membrane transport, then mathematical equations may be formulated for nonlinear drug transport. From the two compartment open model valuable information can be obtained such as

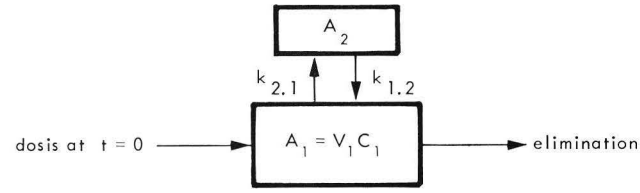


Figure 5.2:

The two compartment open model with rapid bolus intravenous injection where elimination occurs from Compartment no. 1.
A = drug amount; C = drug concentration; V = compartment volume; k = drug transfer.

the volume distribution and the half life of the drug. The volume distribution is simply calculated by:

$$Vd = \frac{dose}{C_0} \tag{5.1}$$

in which C_0 is the concentration of the drug in the blood at time zero. This gives an indication of the volume of the central, well perfused compartment. After a distribution phase representing drug transport to the peripheral compartment, an stationary phase during which the drug is excreted from the body is reached. From the equilibrium phase the t 1/2 (time needed for halving of the drug concentration in the central compartment) can be calculated and employed for the estimation of the duration of effective drug levels (Wagner, 1975). Although this model is often sufficient for poorly diffusable and non-toxic drugs, the complex pharmacokinetics of many anticancer drugs demand the development of multicompartment models (Fig. 5.3), which in contrast to the two compartment model, differentiate between the various subdivisions of well and poorly perfused tissues. Anticancer agents have their specific action based not only on effective blood and tissue levels but also on cell kinetic interactions, which is clear from the specific toxicity to rapidly dividing tissues. An effective drug level is essential in the tumour region, which may be situated anywhere in the central or peripheral compartment. These considerations have led to the design of restricted multicompartment models for Methotrexate, Cytosin-arabinoside and Adriamycin (Bisschoff et al., 1971; Morrison et al., 1975; Chan et al., 1978). Including the well-known assumptions of 1) flow limitation; 2) linear protein binding; and 3) a homogeneous concentration in each compartment, the time history of drug concentration in any compartment of such a model is then:

$$V\frac{dC}{dt} = QC - Q\frac{C}{R} - K\frac{C}{R} - \frac{VmC}{Km + C} \tag{5.2}$$

where C and Cp are the drug concentrations in the compartment and in the plasma, V and Q are the volume and the blood flow rate, R is the distribution ratio between plasma and tissue, K is the apparent first order clearance rate and Vm and Km are the Michaelis-Menten constants for the metabolism in that compartment (Wagner, 1975). The model is described by a number of these equations from time t=0 till the end of the observation period. Thus, the solution of a set of differential equations for one compartment is:

$$\frac{dC}{dt} = c(t) = A\underline{c}(t) \tag{5.3}$$

in which $\underline{c}(t)$ denotes a vector of the compartment drug concentrations

are considerable. Some of these problems can be overcome by including in the model a representation of the degree of uncertainty with which values for the transport rate constants are defined in specific physiologic or pathophysiologic states (Carson and Jones, 1979). Factors responsible for uncertainty include interindividual variability, time variation of the system and variation due to drug effects and environment. Thus, values for a rate constant are assumed to be probalistic more than deterministic. With this approach specific variables like blood flow do not have to be determined separately but are assumed to be as contributing factors to the distribution parameters.

In the case of Adriamycin the usefulness of a multicompartmental model is clearly supported by the toxicity, the triphasic plasma disappearance pattern and the long persistence of the drug (Chapter 3). This indicates that many factors are of influence on the pharmacokinetics of this drug and that the concentration-time history of the drug in each compartment is the result of the particular quality of that compartment such as membrane properties, blood flow,volume and elimination as well as variations in concentration-time histories in all other compartments. This means that the solution of a multicompartmental distribution by a set of independent equations does not approximate the actual situation.

Therefore, a new approach was taken for development of a multicompartment model for the disposition of Adriamycin in the rat, based on parts of the concept of intrinsic biomathematics. This approach assumes the construction of the model to be known, while the numerical values of the drug transfer parameters between compartments are assumed to depend in a complex way on interindividual variability and specific physiologic and pathologic processes. No effort is made to deduce the exact form of these dependencies. In the present case, the drug transfer parameters are interpreted as a set of unknown parameters of a dynamic system (Campello and Cobelli, 1978). When drug concentration time histories of different compartments are experimentally determined, these measurements are interpreted as the response of the dynamic system. Consequently classical system identification techniques may be applied for the estimation of the drug transfer parameters (Eykhoff, 1974). The main objectives of this model are: 1) to identify the actual number of compartments for Adriamycin and to predict the drug disappearance in each of them from known concentrations; 2) to obtain information about drug transfer in the body and to resolve inconsistencies between theory and experimental results; 3) to investigate correlations of drug pertubation of a system and its effects; and 4) to construct it in a way which makes it employable in humans.

5.2 THEORETICAL CONSIDERATIONS

5.2.1 Development of a multicompartment model for Adriamycin

The construction of a pharmacokinetic model is characterized by several assumptions and restrictions which generally determine the complexity and accuracy of the predicted concentration-time histories (Teorell, 1937). A concept which represents physiological significance should include basic physical principles as such, for instance, conservation of drug mass, as well as a reflection of the physiology and anatomy of the subject under consideration.

In view of the major aim of a pharmacokinetic model, which is to predict concentration-time histories in those organs which are not an exact replica of the corresponding time-history in the blood plasma, it is generally composed of a set of interconnected compartments each representing one or more organs or tissues (Wagner, 1975; Campello and Cobelli, 1978). In the model, each compartment represents a particular organ or tissue which is unique with respect to drug uptake and excretion. However, organs and tissues which respond in a more or less similar way to drug administration may be as well modeled by one compartment.

The number of compartments of the model and the way in which they are interconnected determines the so-called structure of the model.

An example of a compartmental model as developed in the course of the present investigation for the distribution of Adriamycin in the rat following intravenous administration is presented in Fig. 5.4. The model consists of 10 separate compartments. The lines represent the pathways of drug transport between individual compartments. Elimination of drug is restricted to the urinary and biliary pathways and assumed to be dependent on the drug quantity in kidneys and liver, respectively. A possible role of the enterohepatic recirculation has been suggested for the disposition of Adriamycin (Benjamin et al., 1973). This physiological redistribution mechanism is not presently included in the model, since it represents a rather small effect and relatively short time periods after drug administration are considered. Longer time intervals, however, may dictate the implementation of recirculation in the model. Since in the rat no significant breakdown of Adriamycin to its metabolites occurs, a conservation of parent drug mass is considered (Chen and Gross, 1979). When this concept is applied, however, to other species or different drugs, metabolic processes should be included by adding additional compartments to the model.

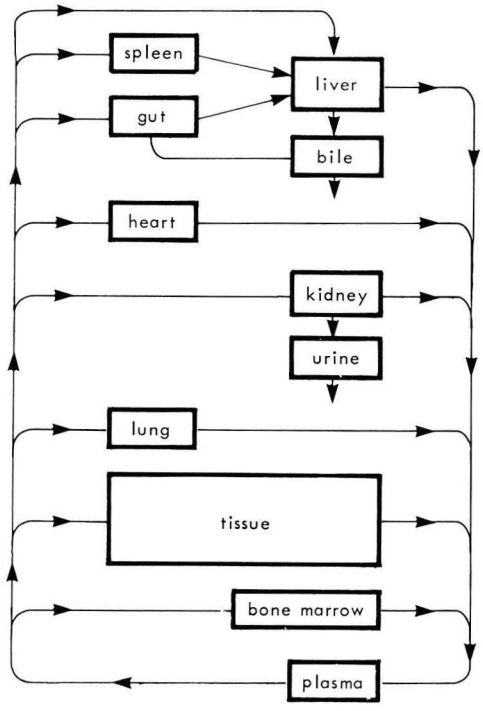


Figure 5.4:

Schematic diagram of the multicompartment open model with rapid intravenous single injection of Adriamycin (7.5 $mg.kg^{-1}$) in the rat. Arrows represent drug mass transfer.

As argued above, the number of compartments is to a certain extent dictated by the uniqueness of the drug concentration-time histories of individual organs or tissues. This is illustrated by the experimental results discussed in Chapter 3. It should not be concluded, however, that each compartment is equally important for the accuracy of the predicted concentration-time histories of the remaining compartments.

The structure of the compartmental model implies a mathematical description once a physical law for the elimination of drug from one to the adjacent compartment has been postulated.

It is postulated that the transport of drug is adequately described by a linear law, i.e., the rate of elimination of drug from a particular compartment to an adjacent compartment is linearly dependent on the quantity of drug in that particular compartment. When taking as an example the kidney compartment, this results directly in

the following differential equation:

$$\dot{A}_5 = k_{5,1} A_1 - k_{1,5} A_5 - k_{9,5} A_5 \tag{5.5}$$

where

$\dot{A}_5$ = rate of change of drug quantity in the kidney
A_1 = drug quantity in plasma
A_5 = drug quantity in kidney
$k_{5,1}$ = drug transfer parameter from plasma to kidney
$k_{1,5}$ = drug transfer parameter from kidney to plasma
$k_{9,5}$ = drug transfer parameter from kidney to urine

Eq. (5.5) can be written in terms of drug concentrations according to:

$$\dot{x}_5 = k_{5,1} \frac{V_1}{V_5} - k_{1,5} x_5 - k_{9,5} x_5 \tag{5.6}$$

where

$\dot{x}_5$ = rate of change of drug concentration in the kidney
x_1 = drug concentration in plasma
x_5 = drug concentration in kidney
V_1 = volume of plasma compartment
V_5 = volume of kidney compartment

Eq. (5.6) is a linear constant differential equation for the concentration of drug in the kidney. Corresponding equations can be derived for the remaining compartments of the model. The complete set of equations has been listed in the appendix.

In those cases where metabolism of Adriamycin is of serious influence on its elimation, constant transfer parameters are not sufficient to adequately describe the distribution of the drug. A saturation model as proposed by Michalis and Menten (1913) may then be essential.

The complete set of differential equations can be interpreted as representing a dynamic system with system state vector $x = \text{col}\ [x_1, x_2, \ldots x_{10}]$ and system matrix A according to

$$\frac{dx}{dt} = Ax \tag{5.7}$$

Eq. (5.7) can be solved when $x(t_0)$, the initial state vector of the system and the system matrix A are known. The solution would consist of the trajectory of the system state vector x, i.e., the time histories of the drug concentration in all of the compartments of the system model. In the present investigations, Adriamycin was administered via a single intravenous bolus injection. A few seconds after administration of the drug in the plasma compartment, it is homogeneously distributed through the plasma, while the concentration in the other compartments is still very close to zero. As a good approximation, therefore all components of the initial state vector are taken as

zero, except that for the first compartment representing the concentration in plasma. The initial plasma concentration can be calculated from the drug dose D and the volume of the plasma compartment V_1:

$$x_1(t_0) = \frac{D}{V_1} \tag{5.8}$$

The elements of the system matrix A are constituted of the compartmental volumes V_i and the rate constants k. In this investigation, the compartmental volumes were experimentally determined. This leaves the rate constants as unknown.

It is shown in the appendix that the rate constants may be estimated from measurements of the drug concentration in several, but not necessarily all, compartments of the model at discrete instants of time after drug administration. The basis of the technique is the so-called model matching principle in which the output of the mathematical model is fitted as well as possible to the experimentally obtained measurements of the system response by selecting an "optimal" set of values for the unknown rate constants k.

Even in the rather hypothetical case of an exact mathematical model, the fit of the model to the measurements will not be perfect due to measurement errors. When these measurement errors are assumed to be random variables, the selection of the "optimal" values for the unknown rate constants may be interpreted as a statistical estimation problem. In the present case, maximum likelihood estimation theory has been applied resulting in maximum likelihood estimates of the unknown rate constants. Once the estimated values of the rate constants are known, the response of the pharmacokinetic system can be calculated and results in:

a) smoothed concentration-time curves of measured drug concentrations at discrete instants of time;
b) predicted concentration-time curves for those compartments from which no drug concentration measurements could be obtained.

An important result is also the estimated variance-covariance matrix of rate constant estimation errors and the variance-covariance matrix of estimation errors of the concentration-time curves as a function of time. Results are presented in the following section.

It should be noted, however, that these results are based upon the following assumptions which are in fact certainly not fully satisfied:
1) the mathematical model is an exact description of reality; and
2) the difference between model output and drug concentration measurements is due only to random, gaussian measurement errors. These assumptions are certainly not fully satisfied due to the following:

a) the mathematical model is only a fairly rough approximation of reality.

b) for drug concentration measurements at each instant of time, a different animal is used: because of biological variation between different animals, the corresponding values of the rate constants will be different and consequently one set of values for the whole set of animals used for the experiments will consequently result in model errors (see section 5.3).

5.3 EXPERIMENTAL INVESTIGATIONS AND MODEL ASSUMPTIONS

The experimental data used for the mathematical modelling of Adriamycin pharmacokinetics were obtained in the female BN rat following a single i.v. bolus injection of 7.5 $mg.kg^{-1}$ and are listed in Table 5.1. Thus distribution data were used of the plasma, the heart, the lungs, the liver, the kidneys, the spleen, the bone marrow and of the biliary and urinary routes of elimination. All data were obtained in rats weighing 165 grams. Since 3 separate experiments failed to demonstrate any drug accumulation in either skeleton or skin, two organs which are poorly diffused, these two organs were subtracted from the tissue compartment which includes those parts of the body in which no determinations of Adriamycin were performed. Wet volumes of organs were defined experimentally in 50 female BN rats aged 12 weeks and are listed in Table 5.1. (see also Table 3.3).

Adriamycin was assumed to be universally dispersed throughout the plasma at the time of administration, which is in fact not entirely valid. No binding of drug to cellular structures or plasma proteins was built into the model and the transport of Adriamycin to other compartments was assumed to be a first order reaction. Since Thin Layer Chromatographic studies demonstrated no metabolic product of Adriamycin during the 8 hours observation period, the drug was supposed to remain unchanged, and a 100 percent recovery from the body was assumed (Chapter 3.3.5).

5.4 RESULTS

Application of the system identification technique as described in the appendix results in maximum likelihood estimates of the rate constants k_{ij} as well as of the smoothed and predicted concentration time histories of the drug concentration. These results are shown in Figs. 5.5 through 5.14. In these Figs. the curves of $\pm$ 1 estimation errors derived from the corresponding diagonal elements of the variance-

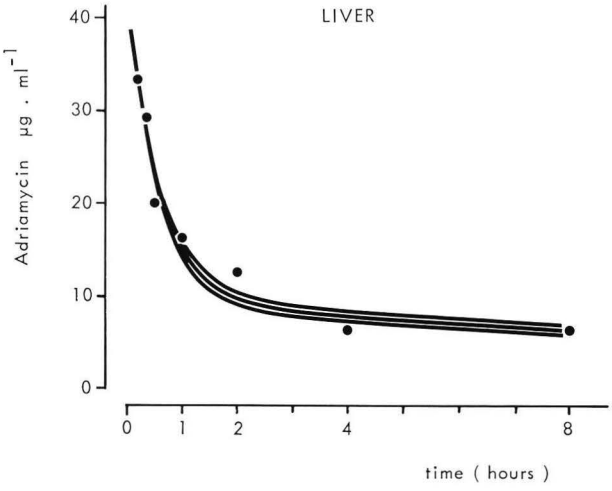


Figure 5.5:

Smoothed disappearance curve and estimation error for Adriamycin in the liver. The actually measured values are indicated in the figure.

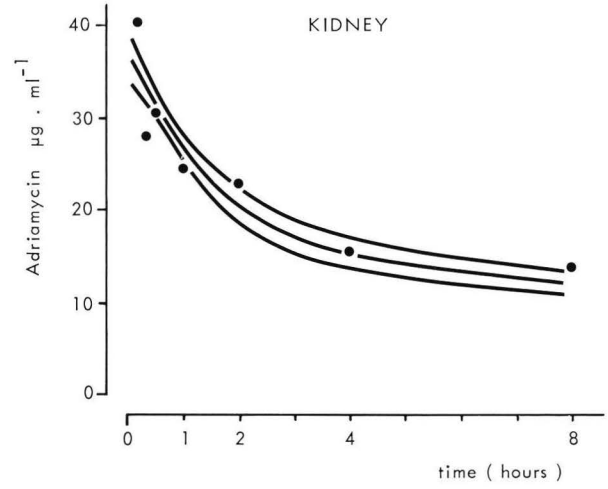


Figure 5.6:

Smoothed disappearance curve and estimation error for Adriamycin in the kidneys. The actually measured values are indicated in the figure.

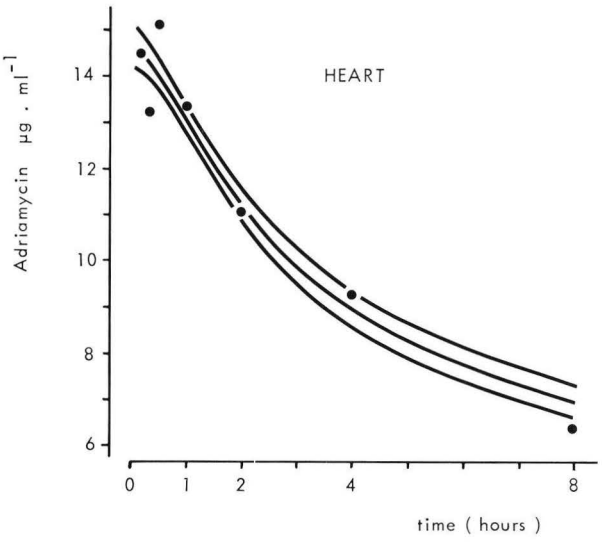


Figure 5.7:

Smoothed disappearance curve and estimation error for Adriamycin in the heart. The actually measured values are indicated in the figure.

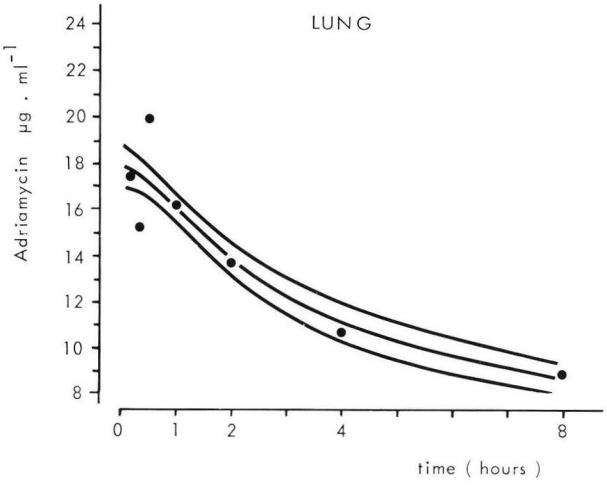


Figure 5.8:

Smoothed disappearance curve and estimation error for Adriamycin in the lungs. The actually measured values are indicated in the figure.

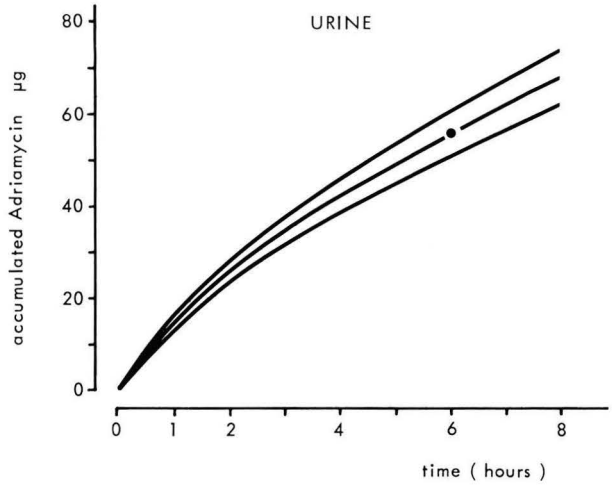


Figure 5.9:
Smoothed accumulated urinary elimination curve and estimation error for Adriamycin. The actually measured values are indicated in the figure.

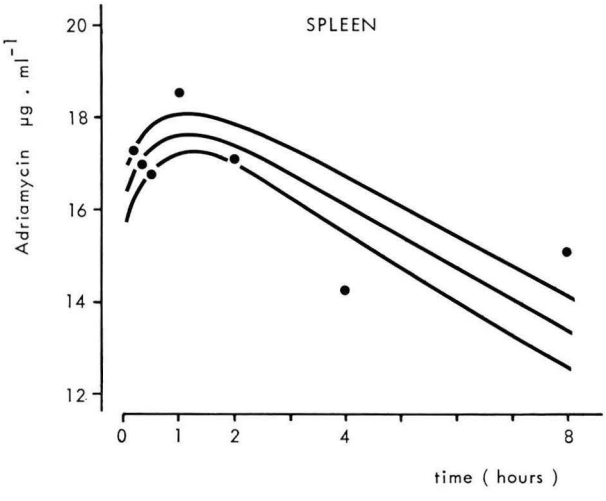


Figure 5.10:

Smoothed disappearance curve and estimation error for Adriamycin in the spleen. The actually measured values are indicated in the figure.

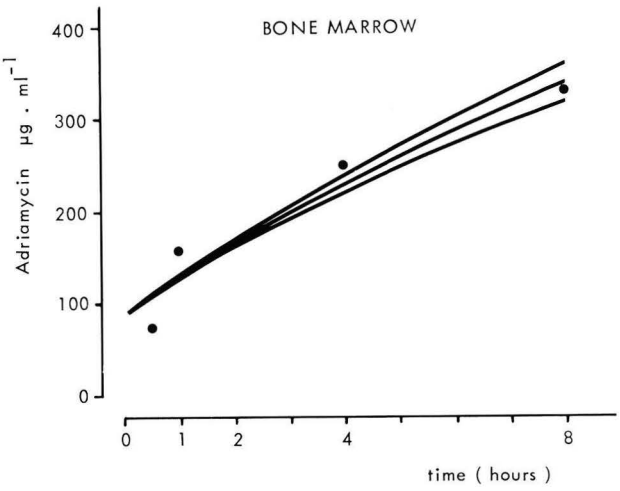


Figure 5.11:

Smoothed disappearance curve and estimation error for Adriamycin in the bone marrow The actually measured values are indicated in the figure.

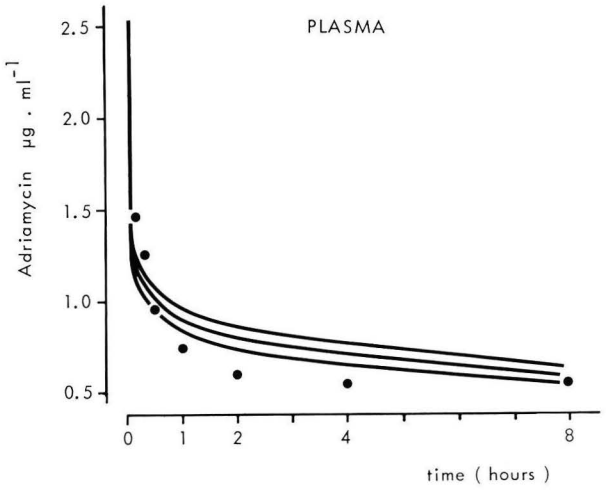


Figure 5.12:

Smoothed disappearance curve and estimation error for Adriamycin in the plasma. The actually measured values are indicated in the figure.

in the blood is indeed bound to some percentage to cellular blood structures. These findings imply that the blood cells have a drug reservoir function, e.g., in the early phase following injection in the plasma drug transfer takes place from the plasma to blood structures, i.e. plasma drug concentrations are higher than whole blood concentrations. Later the situation is reversed: while Adriamycin is released from blood structures to plasma, the plasma concentration is even lower than the whole blood concentration. Since, in the mathematical model no binding of drug to blood cells or plasma proteins was initially assumed, the predicted plasma disappearance curve in fact represents the whole blood disappearance. If the blood cell compartment is separately modeled, a better fit of the smoothed plasma curve to the measured one might be obtained.

Initially the measurements of Adriamycin elimination via the biliary route were not taken into account. Nevertheless, the model prediction compares surprisingly well with what was actually measured (Fig. 5. 13). A possible cause for the slight underestimation could be found in a still hypothetical role of the enterohepatic circle which was not included in the model. As stated in Chapter 5.2, the model smoothes the concentration-time history in those compartments from which experimental data are available. This approach leaves the other, not actually measured drug particles to be present in the tissue com-

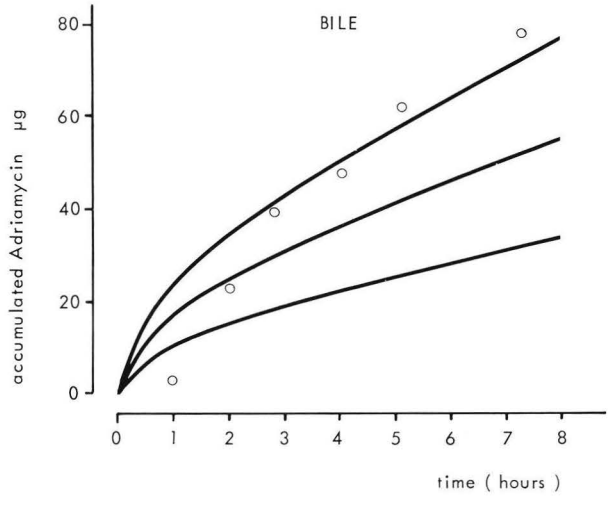


Figure 5.13:

Predicted accumulated biliary elimination curve and estimation error for Adriamycin. The actually measured values, which were not used for the calculation of the model response, are indicated in the figure.

partment which includes the remaining part of the body, or to be eliminated via the bill. The rates of elimination in urine and bile are assumed to be dependent on the drug concentration in kidney and liver, respectively. In the present case, experimental data are available on the Adriamycin concentration in urine, leading to a predicted distribution of the remaining drug quantity over the bile and the tissue compartment (Fig. 5.14).

The smoothed curves of the Adriamycin disappearance in spleen and bone marrow, the two organs of the haemopoietic system, show a pattern which is different from that in other compartments. In spleen, the maximum concentration is reached after 60 minutes, while the drug concentration in bone marrow increases until the end of the observation period. Apparently, this approach for identifying the drug transport parameters permits the existence of compartments in which the drug gradually or even not disappears as would be expected from a pharmacokinetic point of view. This is a relevant characteristic of the model, since cellular and kinetic properties which often differ between compartments, are of influence on the drug behaviour.

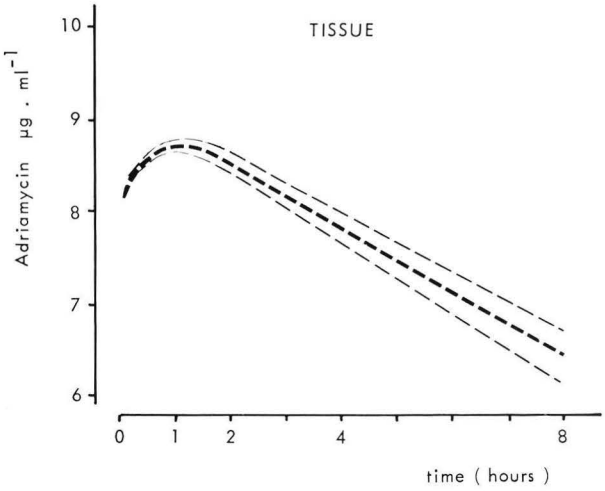


Figure 5.14:

Predicted disappearance curve and estimation error for Adriamycin in the tissue.

5.5 DISCUSSION

Perhaps the major utility of a physiologic model is the potential to predict drug levels in tissues which cannot be readily sampled. A good predictive fit of tissue data would also lend support to the appropriateness of the model design and choice of parameters. Especially if tumours are involved, variations in tissue condition, blood flow and tissue binding in and around the tumour can be expected (see Chapter 3 and Chapter 4). Since the relative contribution of these factors to drug disposition is not easy to determine, new methods of predicting models have to be applied.

Pharmacokinetic models which predict the uptake in and the elimination of a cytostatic agent from a tissue either by the concepts of limited membrane transport or by limited blood flow (Chen and Gross, 1979; Reich et al., 1979) often fail either to predict or to recognize the complex disappearance pattern in rapidly proliferating tissues. In fact these tissues are of special interest with respect to prediction and prevention of toxic drug levels. If special kinetic or cellular characteristics of various tissues are not included in a mathematical model, it appears that prediction only from pharmacokinetic parameters leads to substantial problems. On the contrary, prediction from experimental data by means of identification of the drug transport parameters of all compartments is based on the implementation of all variables contributing to these parameters, thereby revealing the existence of compartments which differ mutually.

As indicated earlier, drug transport from the plasma to other compartments depends partly on the number of drug particles available for uptake. Thus, limitations of the arterial blood flow may limit the uptake of Adriamycin more than do other mechanisms. However, in well perfused organs like the heart, the lungs, the liver and the kidneys, blood flow limitations probably play only a minor role in the drug transport into the cell. This can be concluded from the fact that the concentration-time histories in these organs resemble the plasma curve very much, all exhibiting a triphasic disappearance pattern. Apparently, drug uptake in these organs is either free or is determined by limited membrane transport mechanisms. However, it is possible that other mechanisms, such as diffusion between compartments attribute to drug transport. Without determining the relative contribution of all of these drug transfer factors to the drug uptake, the present model produces an adequate fit in the well-perfused organs.

In comparison to other pharmacokinetic distribution models, the present approach offers new possibilities and problems. Its main ob-

jective is not the simulation of concentration-time histories in various compartments but the identification and interpretation of the drug transfer rate parameters between compartments from experimental data. This leads to absolute information on drug transport between compartments and the drug concentration-time histories. Because the lumped drug transfer parameters are derived from experimentally obtained drug concentrations, they can be assumed to be composed of all factors attributing to drug transport. In this respect this model can be considered to be a more complete and adequate description of reality than models which try to simulate the drug transport by only one or more limitations such as flow, membrane capacities and protein binding. Uppermore it avoids the need to apply rough data on blood flow and membrane transport, which tend to vary interindividually. Application of this concept to the pharmacokinetics of Adriamycin in the rat by maximum likelihood estimation theory results in the most accurate data having the strongest impact on the compartmental system, while less accurate data (having a considerable variation), such as those from spleen and bone marrow, influence the parameter estimation to a lesser extent. Still, any variation in the concentration of Adriamycin in a compartment has an immediate influence on all compartments. It was found, for example, that on purpose reduction of bone marrow numerical values for the Adriamycin concentration leads directly to an increase in predicted biliary excretion. Although it is impossible to exclude biological variations, their impact on prediction of drug concentration is reduced in this way.

Under these conditions and assuming linear kinetics, an organ concentration can be calculated directly from the plasma concentration, e.g., from the model response to an initial dose if the drug transfer parameters have been identified. It is clear that the relation between plasma and organ concentration will be different for each compartment. This is especially evident from the concentration time histories for spleen and bone marrow, which are quite different from those in other compartments.

As stated in Chapter 3 pathological conditions may alter this relationship to unknown proportions. To determine the altered relationship, drug measurements in some compartments and new estimations on the volume of pathologically deteriorated organs are needed to find the new drug transfer parameters and to estimate the new state trajectory. This can be performed with the same mathematical approach by estimation of the drug transfer parameters which are expected to be changed, for example by altered vascularization or tumour cell infiltration. Subsequently, some measured concentrations of Adriamycin in

easily accessible compartments like blood, urine and bone marrow are introduced and the drug disappearance curves (new state trajectory) can be found.

Especially the influence of tumours on distribution ratios (Hoeschele and van Camp, 1972) should be implemented by simulation and identification of drug transfer rates obtained from experimental data of tumour bearing animals and man.

Application of this concept to clinical pharmacokinetics is possible if limitations are made. As for all mathematical models developed in animals, the rate of equivalence between animals and man is speculative (Wagner, 1975). However, the ultimate response of the mathematical model described here is not dependent on technical data on blood flow and can be easily checked for reliability, since all compartments together determine the state trajectory. Thus, systematic mistakes are readily recognized.

When assuming no basic differences to exist between rat and man, application may be performed by simple supplementation of the values for weight and volume. However, the occurrence of metabolic degradation of Adriamycin in humans (Benjamin et al., 1973; Watson and Chan, 1976) urges the implemention of mathematical formulations for this way of drug elimination, for example, with the Michaelis-Menten constants (Michaelis and Menten, 1913). Introduction of these constants into the model also allows its application to drugs which are known to be heavily metabolized.

Regarding the validation of pharmacokinetic modeling in the clinical context, additional procedures may be worth to be undertaken. Of special significance to the clinician are features like delays and overshoots in the drug distribution rather than a detailed study of the entire model response. Where alternative hypotheses arise as to the structure of the compartmental system, crucial experiments should then be designed to reject a structure by its failure to reproduce the expected patterns of response.

It may be concluded that the present approach for the determination of drug transfer parameters offers significant advantages in the description and analysis of drug distribution. Application of maximum likelihood theories avoids the implemention of unaccurate physiological data and facilitates the solution of differential equations for drug transfer. Uppermore, it allows the optimal use of information being present in pharmacokinetic data.

5.6 APPENDIX

MAXIMUM LIKELIHOOD ESTIMATION OF SYSTEM PARAMETERS AND SYSTEM RESPONSE

The assumption that drug excretion into a compartment i is proportional in a 10 compartment model to the drug quantity or concentration in the adjacent compartment j leads with the structure of the compartmental model as shown in Fig. 5.4, directly to the following set of linear constant first order differential equations:

$$\dot{x}_1 = -(k_{2,1} + k_{3,1} + k_{4,1} + k_{5,1} + k_{6,1} + k_{7,1} + k_{10,1})\, x_1 + k_{1,2} \frac{V_2}{V_1} x_2 + k_{1,4} \frac{V_4}{V_1} x_4 + k_{1,5} \frac{V_5}{V_1} x_1 + k_{1,6} \frac{V_6}{V_1} x_6 + k_{1,7} \frac{V_7}{V_1} x_7 + k_{1,10} \frac{V_{10}}{V_1} x_{10} + \frac{1}{V_1} u \quad \text{(PLASMA)}$$

$$\dot{x}_2 = k_{2,1} \frac{V_1}{V_2} x_1 - (k_{1,2} + k_{8,2})\, x_2 + k_{2,3} \frac{V_3}{V_2} x_3 \quad \text{(LIVER)}$$

$$\dot{x}_3 = k_{3,1} \frac{V_1}{V_3} x_1 - k_{2,3}\, x_3 \quad \text{(SPLEEN)}$$

$$\dot{x}_4 = k_{4,1} \frac{V_1}{V_4} x_1 - k_{1,4}\, x_4 \quad \text{(HEART)}$$

$$\dot{x}_5 = k_{5,1} \frac{V_1}{V_5} x_1 - (k_{1,5} + k_{9,5})\, x_5 \quad \text{(KIDNEY)}$$

$$\dot{x}_6 = k_{6,1} \frac{V_1}{V_6} x_1 - k_{1,6}\, x_6 \quad \text{(LUNG)}$$

$$\dot{x}_7 = k_{7,1} \frac{V_1}{V_7} x_1 - k_{1,7}\, x_7 \quad \text{(TISSUE)}$$

$$\dot{x}_8 = k_{8,2} \frac{V_2}{V_8} x_2 \quad \text{(BILE)}$$

$$\dot{x}_9 = k_{9,5} \frac{V_5}{V_9} x_5 \quad \text{(URINE)}$$

$$\dot{x}_{10} = k_{10,1} \frac{V_1}{V_{10}} x_1 - k_{1,10}\, x_{10} \quad \text{(BONE MARROW)}$$

in which:

x_i = drug concentration in compartment i;
u = intravenous drug administration;
V_i = volume of comparment i;
$k_{i,j}$ = rate constant of drug concentration from compartment j to compartment i.

Because Adriamycin is administered during a relatively short time interval, the input signal can be adequately approximated by an impuls at time t = 0. Consequently, the response of the above set of differential equations with initial condition $x_i(0) = 0$ for i = 1, ..., 10 is identical to the response on initial condition $x_1(0) = \frac{u(0)}{V_1}$, $x_i(0) = 0$ for i = 2, ..., 10 and u(t) = 0 for $t > 0$.

The rate constants $k_{i,j}$ in the system of differential equations listed above are in principle unknown. When a set of arbitrary values for the rate constants such that $k_{i,j} \geqslant 0$, all i, all j and $x_i(t) \geqslant 0$, all i, all t are substituted, the system of differential equations can be solved resulting in drug concentration vs time curves of the constituting compartments. At a given set of discrete instants of time after drug administration these calculated concentrations are compared to corresponding measured concentrations of the real system response. Subsequently a performance index for the fit of the model to the measurements is defined as a weighted sum of the squares of the model-real system differences. As a last step "optimal" values of the rate constants are calculated by minimizing the performance index with respect to the rate constants. Thus the differences between model and real system response are minimized in the least squares sense. The resulting nonlinear optimization problem has been solved by applying different numerical optimization schemes as Newton Raphson (Eijkhoff, 1974) and the direct optimization method of Powell (Brent, 1973). The applied "model matching" procedure can also be interpreted in terms of maximum likelihood estimation theory. This leads to not only "estimated" values of the rate constants but also to the possibility to calculate the corresponding estimation errors of the rate constant as well as of the resulting model concentration time histories (Mulder et al., 1979). Results are shown in Figs. 5.5 - 5.14 and Table 5A.

Table 5A

ESTIMATED RATE CONSTANTS (min^{-1}) AND ESTIMATION ERRORS (SD %)

$k_{2,1}$	0.2816 ± 0.1	$k_{1,5}$	0.0074 ± 27.8
$k_{3,1}$	0.0073 ± 3.8	$k_{1,6}$	0.0104 ± 12.4
$k_{4,1}$	0.0114 ± 3.3	$k_{8,2}$	0.0023 ± 42.5
$k_{5,1}$	0.0533 ± 8.0	$k_{7,1}$	1.1960 ± 0.02
$k_{6,1}$	0.0343 ± 5.8	$k_{1,7}$	0.0066 ± 9.2
$k_{3,2}$	0.0064 ±10.3	$k_{9,5}$	0.0076 ± 11.4
$k_{1,2}$	0.0294 ± 3.6	$k_{10,1}$	0.1327 ± 0.2
$k_{1,4}$	0.0106 ± 9.0	$k_{1,10}$	0.00001 ± 3299.4

CHAPTER 6

GENERAL DISCUSSION

Human society in the Western World is at present greatly influenced by the calculation of risk factors and the economic relevance of avoiding them. This situation is the result of both increasing scientific information and the economical power which permit the avoidance of dangerous situations. Still, in medicine, there is a daily confrontation with risk factors, which cannot be avoided. The outcome of therapeutic approaches often is determined by side effects, which up to now have been more frequently antagonized with empirism than with calculation. Generally research on side effects of a treatment modality starts shortly after its introduction. However, much of the information is empirical, e.g., patient symptoms are used to judge the intrinsic value of the treatment. This is especially true for cancer chemotherapy, where the major obstruction in establishing better therapeutic regimens is toxicity. Extensive investigations on the molecular basis and the *in vivo* characteristics of cytotoxic agents did not start before the seventies. Pharmacokinetics of these drugs are mostly unknown or not employed to design better dose schedules. Still, the only way to decrease toxicity and to increase the therapeutic index of cytostatic drug starts with obtaining information on their characteristics.

The cytotoxic activity of Adriamycin explains its severe harmful effcts on several normal tissues. This problem, also existing with many other anticancer drugs, forces to investigate the distribution kinetics of the agent in order to learn whether the efficacy and the toxicity of the drug is concentration-dependent and whether manipulations of the dosage regimen may alter the therapeutic index. Although the clinical manifestations of toxicity are easily recognizable, it is impossible to investigate the underlying pharmacokinetic phenomena in patients without life-threatening manipulations. Therefore, animal models are considered suitable to investigate drug kinetics in all parts of the body *in vitro* and *in vivo*. Up to now, there have been several studies on the distribution of Adriamycin in animals and few human autopsy tissue data are available. These animal studies have mainly been carried out in normal rats and mice, although Adriamycin

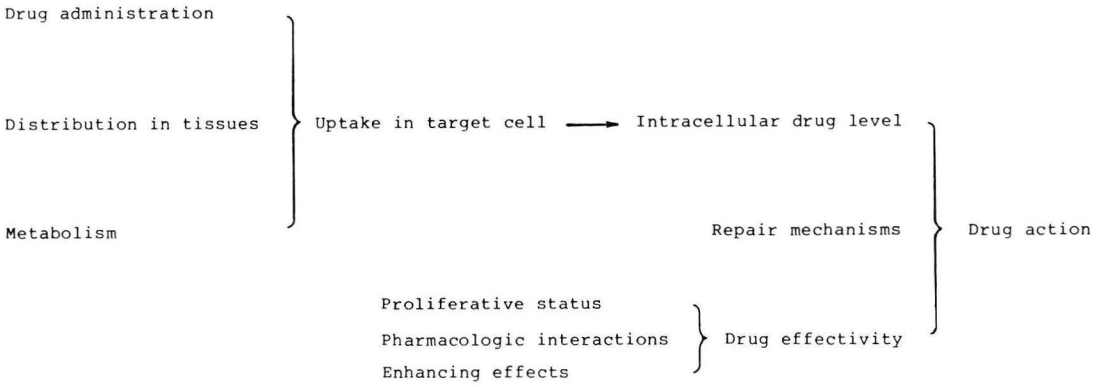
is restrictively administered to tumour bearing patients. The observed differences in tissue distribution between BN and BNML rats indicate that the presence of a rapidly proliferating tumour cell compartment essentially alters drug kinetics, a phenomenon which naturally is of influence on toxicity. In fact, the concentrations of Adriamycin in leukaemia infiltrated tissues are significantly lower when compared with those in the same tissues when no leukaemia cells are involved, probably as a function of tumour load. Simultaneously, the urinary elimination of Adriamycin is decreased due to impaired kidney excretory functions, as can be concluded from plasma creatinin levels. The reduced uptake of Adriamycin in leukaemia infiltrated organs consequently results in higher drug concentrations in other tissues for a prolonged period, as can, for example, easily be observed from the plasma disappearance curve. On this account, plasma drug levels cannot merely be considered to be representive of tissue concentrations as is presently done in some clinics. From the distribution studies it appears that especially information on bone marrow concentrations may not be gained from plasma sample analysis. This illustrates a potential clinical risk of underestimating haematological toxicity from the plasma drug disappearance.

Basic information is needed on the dose-effect relationship between Adriamycin and bone marrow cells. The in vitro data from the present study, emphasizing the different sensitivity of leukaemic and normal bone marrow cells, indicate the harmful effects of high concentrations of Adriamycin. As discussed BNML cells take up the drug to a greater extent and are more sensitive than haemopoietic cells. However, in vivo Adriamycin accumulates in normal bone marrow more than in leukaemic bone marrow, although in both cases to an extent which equally harms leukaemic and haemopoietic cells. Apparently, the histological structure of leukaemia infiltrated bone marrow tissue inhibits free penetration of Adriamycin, which leads to lower intracellular drug concentrations in spite of the superior properties of BNML cells regarding the uptake of Adriamycin. Essentially the same observations are made in spleen. Evaluation and combination of in vivo and in vitro data leads to the conclusion that in the BNML rat the presently employed in vivo dose of Adriamycin is too high and should be halved, if toxicity is to be avoided. In view of the resemblance of this model to human AML, these findings may serve to justify decreases in clinical dosages of Adriamycin.

Fig. 6.1 summarizes some important physiological mechanisms contributing to the ultimate drug effect. From this figure, it appears clearly that an estimation of the drug effect cannot be made by simple

Figure 6.1

DETERMINANTS OF DRUG ACTION ON CELLS



determination of just one of the causal factors. However, clinical practice insufficiently investigates these parameters in patients, due to several problems including anatomical inaccessibility of the body region under consideration. In recent years, some early attempts have been made to approach this problem in a mathematical way. These simulations were designed to predict response to ara-C, from pharmacokinetic and cell kinetic data in the L1210 mouse leukaemia (Morrison et al., 1975). At present, however, it is still not yet possible to incorporate too many variables in mathematical simulation models due to insufficiency of computer programs. Therefore, in this study, a start was made with an alternative approach which analyzes biological data and thereafter derives the unknown drug transfer parameters from them. The pharmacokinetics of Adriamycin in normal animals were subjected to analysis and a next step will be the distribution in leukaemic animals, which is, of course, more realistic for comparison with AML patients. The same approach might be employed for the analysis of cell kinetic parameters especially how they change after therapy.

Although the concept of the application of maximum likelihood theories to the solution of unknown transfer parameters is still in a developmental stage, it can be concluded that it offers a new possibility to analyze biological data. A great advantage is its capacity to separate hard and soft data by comparing both in one system. Additionally, it clearly indicates its limitations by a consequent lack of fitting results if wrong assumptions are made. The relevance of such an approach is illustrated by the problems encountered in comparing predicted and experimental results. For example, in the present study it appeared that the mathematical model could not predict bone marrow concentrations adequately when the organ concentration was assumed to be solely dependent on drug levels of arterial and venous blood. It proved necessary to assume that the output of Adriamycin from the bone marrow could be neglected in order to simulate the actual situation where the drug remains accumulated in killed cells. Therefore, it may be concluded that the major power of the model is its physiological resemblance to AML and its capacity to maximize the information that can be drawn from drug distribution data.

The observation that the spleen and bone marrow which, are the major haemopoietic organs, show a concentration-time history different from that in all other organs indicates that it is probably unrealistic to investigate the pharmacokinetics of cytostatic drugs in plasma only, except in those clinical situations where a clear relationship exists between plasma levels and toxicity. At present, such relationships with this drug have not yet been found in humans. This study

indicates that the concentration-time histories in several organs are differently related to that in plasma and are subject to variations where leukaemia is involved. Consequently, plasma samples only are insufficient for monitoring the disappearance of Adriamycin from the body and should be employed to avoid toxicity only if additional information from tissue accumulation is available.

Furthermore, invitro assessment of the drug sensitivity of normal and leukaemic bone marrow cells may serve to determine optimal concentrations in the haemopoietic compartment. Combination of these in vitro data and in vivo drug distribution affords a rational basis for chemotherapy. This may be performed with little effort since the technique described here for measuring the ^{3}H-TdR uptake is easy and rapid. Such an approach limits chances for toxicity and consequently improves the therapeutic index to a higher degree than might be with randomization of the dosage of Adriamycin, as done in clinical protocols.

SUMMARY

The optimal protocol for the treatment of acute myeloid leukaemia presently consists of combination chemotherapy including one of the anthracyclin cytostatic drugs Adriamycin or Daunomycin. One of the most serious drawbacks of these drugs, however, is the fact that they exhibit toxicity to the haemopoietic system, which may lead to premature termination of the treatment. The haematotoxicity of Adriamycin is a disadvantage especially when applied in patients with acute myeloid leukaemia, who often show haemopoietic disorders due to infiltration and suppression of the bone marrow by leukaemic cells. Clinically, it has been found that inhibition of the haemopoietic activity by Adriamycin depends on the dose and the frequency of administration. Therefore, it is necessary to gain insight into the distribution of Adriamycin in the body and to investigate its effect on normal haemopoietic and on leukaemic cells, as described in the introductory Chapter 1.

Although a considerable amount of pharmacokinetic knowledge of Adriamycin is already available, it is mainly restricted to healthy animals. This was a major reason to initiate this study which aims at elucidating the pharmacokinetics of the drug in rats bearing an experimental acute (pro)myelocytic leukaemia. As an introduction to the vast literature on the in vivo and in vitro effects of Adriamycin, the most relevant data are summarized in Chapter 2.

Adriamycin is a product of Streptomyces peucetius and exerts its cytostatic action by being irreversibly bound to nuclear DNA and inhibition of the enzymes DNA and RNA polymerase which are essential for cell replication. It is clear that these effects depend on the bioavailability of Adriamycin, which is determined by the dose, the route of administration and the metabolism, which vary between species. Therefore, several analytical procedures have been developed in order to make it possible to determine the content of Adriamycin in biological fluids and tissues. Application of these procedures has demonstrated that the drug is rapidly cleared from the plasma and accumulates in the tissues for more than 24 h, in both animals and man. The question whether the toxicity of Adriamycin is the result of selective accumulation in some tissues is investigated and discussed in Chapter 3.

The pharmacokinetics of the drug following a clinical dose differ considerably between healthy and leukaemic animals, especially in

those organs which have been infiltrated by leukaemic cells. These differences lead to a different pattern in leukaemic animals when compared with their healthy equivalents. A possible explanation for the severe haematotoxicity of the drug is found in the extremely high drug concentrations found during more than 8 h in bone marrow. It is remarkable that the concentrations reached in normal bone marrow are twice those in leukaemic bone marrow. This difference may be the result of at least two phenomena, the specific characteristics of haemopoietic and leukaemic cells regarding the uptake of Adriamycin and a change in the penetration of the drug into the bone marrow space. Based on these observations it may be concluded that an estimation of the concentration in bone marrow cannot be made from the plasma concentration if no information is available on the relative number of leukaemic cells in the bone marrow.

In Chapter 4 *in vitro* experiments which focus on the effects of Adriamycin on haemopoietic bone marrow cells and leukaemic cells isolated from bone marrow and spleen of leukaemic rats are discussed. The DNA and RNA syntheses in leukaemic cells are inhibited with 40% by low concentrations of Adriamycin which do not inhibit these cell functions in normal bone marrow cells, while high drug concentrations have equal effects on the two cell types. These differences are also found if the clonogenic bioassays Leukaemia Colony Forming Unit spleen (for leukaemic cells) and Colony Forming Unit spleen (for haemopoietic cells) are used as criteria for the cytostatic action of Adriamycin. Both experimental procedures demonstrate that low concentrations of the drug ($0.01 - 1.0$ ug.10^{-7} cells) harm mainly leukaemic cells, while higher concentrations ($5.0 - 50.0$ ug.10^{7} cells) kill normal haemopoietic and leukaemic cells at the same rate. It should be noted that the latter concentrations are in a range which is obtained *in vivo* in bone marrow if a clinical dosage of Adriamycin is administered.

In Chapter 5, the development of a mathematical model for the *in vivo* distribution of Adriamycin in the rat is discussed. Such a model is required to analyse the concentration time histories of the drug in various organs (compartments) and the relationships among them. For this purpose, a 10-compartment model has been constructed in which all compartments are assumed to be part of a system which receives an input signal (drug administration) of which the course is identified with implementation of experimental data. With this approach, optimal concentration time histories and their accuracy are obtained for all compartments of the model, including those for which either no or only few biological data are known. A major advantage of this model is the close agreement between smoothed and experimental data. Secondly, it

offers a new possibility to compare experimental and clinical drug distribution data which may lead to more accurate interpretation of drug kinetics in individual patients.

In Chapter 6, the results are discussed in coherence and promising leads are tentatively extrapolated to man.

SAMENVATTING

De optimale behandeling van acute myeloide leukemie bestaat uit een gecombineerde therapie met meerdere cytostatica, waarvan Adriamycine of het verwante Daunomycine deel uitmaken. Een nadeel van deze zogenaamde combinatie-chemotherapie is echter het feit dat bij een groot aantal van de patiënten ernstige bijwerkingen optreden, die kunnen leiden tot voortijdige beëindiging van de behandeling. Met name een sneldelend weefsel als het beenmerg, waar de normale hemopoiese plaats vindt, is zeer gevoelig voor de cytotoxische werking van Adriamycine. Hierdoor treedt tijdens de behandeling vaak een sterke remming op van de hemopoiese, die bij leukemiepatiënten toch al verstoord is tijdens het spontane verloop van de ziekte. Deze beenmergremming is afhankelijk van de toegediende dosis Adriamycine. Daarom is het noodzakelijk, zoals ook in Hoofdstuk 1 beschreven wordt, om inzicht te verkrijgen in de distributie van Adriamycine in het lichaam en het effect er van op beenmergcellen te kwantificeren.

Hoofdstuk 2 geeft een overzicht van de huidige kennis inzake het werkingsmechanisme en de farmacokinetiek van Adriamycine. Dit door de schimmel Streptomyces peucetius geproduceerde cytostaticum oefent zijn remmende werking op de celdeling uit door de tot standkoming van een irreversibele binding met de base paren, die deel uitmaken van het DNA molecuul. Hierdoor wordt indien voldoende Adriamycine aanwezig is de replicatie en splitsing van DNA, die essentieel zijn voor de celdeling, voorkomen. Het is duidelijk dat de beschikbaarheid van Adriamycine hiervoor een kritische factor is, hetgeen duidelijk blijkt uit het feit dat het uiteindelijke effect zich omgekeerd verhoudt tot de snelheid waarmee de stof gemetaboliseerd wordt. Dit metabolisme en de snelheid er van varieert met de toegediende dosis en de diersoort. Daarom zijn verscheidene methodes ontwikkeld om het gehalte aan Adriamycine in dierlijke weefsels te bepalen. Toepassing hiervan heeft aangetoond dat Adriamycine zich snel in de weefsels verspreidt en daar gedurende minstens 24 uur aantoonbaar blijft. Het ontbreekt echter aan gedetailleerde waarnemingen, betreffende de vraag of de selectieve toxiciteit van dit middel gecorreleerd is met de concentraties die in sommige weefsels bereikt worden. Deze vraagstelling wordt besproken en onderzocht in Hoofdstuk 3.

Teneinde de werkelijke situatie zo dicht mogelijk te benaderen, is de farmacokinetiek van Adriamycine bestudeerd in normale ratten, maar tevens in ratten die lijden aan een vorm van acute myeloide leukemie

(BNML), die in vele opzichten overeenkomsten vertoont met de menselijke acute myeloide leukemie (Hagenbeek, 1977). Na toediening van een dosis, die vergelijkbaar is met de klinisch gebruikte hoeveelheid, blijkt Adriamycine snel uit het bloed te verdwijnen en zich te stapelen in onder andere hart, longen, lever, milt, nieren en beenmerg. Met name in het beenmerg is gedurende lange tijd een zeer hoge concentratie aantoonbaar, hetgeen een verklaring kan zijn voor de waargenomen toxische verschijnselen in dit orgaan. Een significant verschil in concentratie blijkt aanwezig te zijn tussen organen afkomstig van normale en van leukemische ratten, met name als het organen betreft (lever, milt, beenmerg) die in het geval van leukemische dieren sterk geinfiltreerd zijn door tumorcellen. Dit verschil kent twee belangrijke oorzaken, n.l. de specifieke eigenschappen van leukemische cellen betreffende de opname van Adriamycine, en veranderingen in de bloedvoorziening van die organen, die als gevolg van infiltratie met leukemische cellen sterk toegenomen zijn in celdichtheid. Daarentegen zijn het concentratieverloop en de maximale concentratie gelijk in normale en leukemische ratten. Dit indiceert dat een mogelijke conclusie inzake de geschatte concentratie in een orgaan niet te trekken is op grond van gegevens betreffende de plasmaconcentratie, zolang niet bekend is of en hoeveel leukemische cellen aanwezig zijn binnen dat orgaan.

Als aanvullend onderzoek naar de oorzaken van genoemde verschillen zijn *in vitro* experimenten verricht inzake de gevoeligheid van hemopoietische en leukemische beenmerg- en miltcellen voor Adriamycine. Zoals in Hoofdstuk 4 beschreven is, blijkt een dosis-afhankelijk effect van Adriamycine te kunnen worden waargenomen. In termen van remming van de DNA- en RNA-synthese en vermindering van het aantal clonogene cellen blijken leukemische cellen gevoelig te zijn voor Adriamycine bij lagere concentraties dan geldt voor hemopoietische cellen. Dat dit verschil te wijten is aan de specifieke gevoeligheid van de beide celtypes, blijkt uit het feit dat geringe verschillen bestaan inzake de hoeveelheid opgenomen Adriamycine in de cellen. De mogelijkheid om de waargenomen verschillen te gebruiken om de therapeutische waarde van Adriamycine te verhogen, wordt besproken in het licht van de relatie tussen *in vitro* gevoeligheid en *in vivo* bereikte concentraties.

In Hoofdstuk 5 wordt de ontwikkeling besproken van een mathematisch model voor de *in vivo* distributie van Adriamycine in de rat. Dit model is noodzakelijk gebleken aangezien complexe pharmacokinetische gegevens moeilijk te interpreteren zijn, indien geen indruk aanwezig is omtrent de onderlinge relaties van concentratieverlopen in ver-

schillende organen (= compartimenten). Het hier besproken model kent 10 verschillende compartimenten, waarin het concentratieverloop van Adriamycine berekend wordt vanuit de wiskundige benadering dat alle compartimenten met elkaar samenhangen en elkaar beinvloeden. Dit nieuwe concept is uitgewerkt met behulp van systeemidentificatie- en schattingstheoriën op een digitale computer. De nauwkeurigheid van voorspelde concentraties, en hun verloop in tijd, ten opzichte van de waargenomen concentraties is met toepassing van genoemde technieken aanzienlijk groter dan met conventionele multicompartimentenmodellen. De waarde van deze benadering ligt tevens in de eenvoudigere toepassing in de klinische situatie en in het vergelijkbaar maken van proefdiergegevens met die van de mens.

In Hoofdstuk 6 worden de resultaten in samenhang besproken en wordt voorzichtig gespeculeerd over de mogelijke klinische toepassingen.

REFERENCES

Aglietta, M. and Colly, L.P.: The relevance of recruitment-synchronization in the scheduling of ara-C in a slow growing acute myeloid leukemia of the rat. Cancer Res. 39, 1979, 2727.

Aglietta, M. and Sonneveld, P.: The relevance of cell kinetics for optimal scheduling of 1-β-D-arabinofuranosylcytosine and methotrexate in a slow growing acute myeloid leukemia (BNML). Cancer Chemother. Pharmacol. 1, 1978, 219.

Andersson, B., Beran, M., Eberhardsson, B., Eksborg, S., Slanina, P.: Uptake distribution of Daunorubicin and Daunorubicin-DNA complex in mice as studied by whole body autoradiography and liquid chromatography. Cancer Chemother. Pharmacol. 2, 1979, 159.

Arena, E., D'Alessandro, N., Dusonchet, L. et al.: Analysis of the pharmacokinetic characteristics, pharmacological and chemotherapeutic activity of 14-hydroxydaunomycin, a new drug endowed with antitumor activity. Arzneim. Forsch. 24, 1971, 1258.

Armacone, F., Franceschi, G., Penco, S. et al.: Adriamycin (14-hydroxydaunomycin), a novel antitumor antibiotic. Tetrahedron Lett. 13, 1969, 1007.

Armacone, F., Cassinelli, G., Franceschi, G. et al.: Structure and physicochemical properties of adriamycin (doxorubicin). In: Intern. Symp. on Adriamycin (Eds. S.K. Carter, A. DiMarco, M. Ghione et al.). Springer Verlag, New York, 1972, p. 9.

Barlogie, B., Drewinko, B. and Hart, J.S.: Cytokinetic effects of adriamycin (ADR) in a human lymphoma cell line. Proc. Am. Assoc. Cancer Res. and ASCO 16, 1975, 23.

Bachur, N.R., Moore, A.L., Bernstein, J.G. and Liu, A.: Tissue distribution and disposition of daunomycin (NSC-82151) in mice: Fluorometric and isotopic methods. Cancer Chemother. Rep. 54, 1970, 89.

Bachur, N.R. and Gee, M.: Daunorubicin metabolism by rat tissue preparations. J. Pharmacol. Exptl. Therap. 177, 1971, 567.

Bachur, N.R. and Gee, M.: Oxygen sensitive reductive cleavage of daunorubicin and adriamycin. Fed. Proc. 31, 1972, 835.

Bachur, N.R., Egolin, M.J. and Hildebrand, R.C.: Daunorubicin and adriamycin metabolism in the golden Syrian hamster. Biochem. Med. 8, 1973, 352.

Bachur, N.R., Hildebrand, R.C. and Jaenke, R.S.: Adriamycin and daunorubicin distribution in the rabbit. J. Pharmacol. Exptl. Therap. 191, 1974, 331.

Bachur, N.R.: Adriamycin (NSC-123127) pharmacology. Cancer Chemother. Rep. 6, 1975, 153.

Bachur, N.R., Steele, M., Meriwether, W.D. and Hildebrand, R.C.: Cellular pharmacodynamics of several anthracycline antibiotics. J. Med. Chem. 19, 1976, 651.

Bachur, N.R. and Gee, M.: Microsomal reductive glycosidase. J. Pharmacol. Exptl. Therap. 197, 1976, 681.

Barlogie, B., Drewinko, B., Johnston, D.A. and Freireich, E.J. The effect of adriamycin on the cell cycle traverse of a human lymphoid cell line. Cancer Res. 36, 1976, 1975.

Bekkum, D.W. van, Oosterom, P. van and Dicke, K.A. In vitro colony formation of transplantable rat leukemias in comparison with human acute myeloid leukemia. Cancer Res. 36, 1976, 941.

Benjamin, R.S., Riggs, C.E. and Bachur, N.R.: Pharmacokinetics and metabolism of adriamycin in man. Clin. Pharmacol. Ther. 14, 1973, 592.

Benjamin, R.S., Riggs, C.E., Serpick, A.A. et al.: Biliary excretion of adriamycin (A) in man. Clin. Res. 22, 1974, 483.

Benjamin, R.S.: Clinical pharmacology of adriamycin (NSC-123127). Cancer Chemother. Rep. 6, 1975, 183.

Benjamin, R.S.: A practical approach to adriamycin (NSC-123127) toxicology. Cancer Chemother. Rep. 6, 1975, 191.
Berg, K.J. van den: The role of amino acids in the mitogenic activation of lymphocytes. Thesis. Leiden University, 1974.
Bisschoff, K.B., Dedrick, R.L. and Zaharko, D.S.: Preliminary model for methotrexate pharmacokinetics. J. Pharm. Sci. 59, 1970, 149.
Bisschoff, K.B., Dedrick, R.L., Zaharko, D.S. and Longstreth, J.A.: Methotrexate pharmacokinetics. J. Pharm. Sci. 60, 1971, 1128.
Bisschoff, K.B.: Some fundamental considerations of the applications of pharmacokinetics to cancer chemotherapy. Cancer Chemother. Rep. 59, 1975, 777.
Bonadonna, G., Rossi, A., Valagussa, P., Banli, A. and Veronesi, U. The CMF program for operable breast cancer with positive axillary nodes: updated analysis on the disease-free interval, site of relapse and drug tolerance. Cancer 39, 19.., 2094.
Bosmann, H.B. and Kessel, D.: Inhibitors of an RNA-dependent DNA polymerase. FEBS Lett. 15, 1971, 273.
Brent, R.P.: Algorithms for minimization without derivatives. Prentice Hall, Inc., Englewood Cliffs, N.J., 116-167, 1973.
Campello, L. and Cobelli, C.: Parameter estimation of biological stochastic compartment models. An application. IEEE Trans. Biomed. Eng., BME-25, 1978, 139.
Carson, E.R. and Jones, E.A.: Use of kinetic analysis and mathematical modeling in the study of metabolic pathways in vivo. New Engl. J. Med. 18, 1979, 1016.
Carter, S.K.: Adriamycin, a review. J. natl. Cancer Inst. 55, 1975, 1265.
Carter, S.K., Bahowski, M.T. and Hellmann, K.: Chemotherapy of cancer. J. Wiley and Sons, New York, 1977.
Casazza, A.M., Gambarrucci, C. and Silvestrini, R.: Effects of daunomycin and adriamycin on the murine sarcoma virus (Moloney) in vitro. Rev. Eur. Etud. Clin. Biol. 17, 1972, 622.
Dedrick, R.L., Forrester, D.D. and Ho, K.H.W.: In vitro - in vivo correlation of drug metabolism - deamination of 1-D-arabinofuranosylcytosine. Biochem. Pharmacol. 21, 1972, 1.
Dedrick, R.L., Forrester, D.D., Cannon, J.N. et al.: Pharmacokinetics of 1- β -D-arabinofuranosylcytosine (ara-C) deamination in several species. Biochem. Pharmacol. 22, 1973a, 2405.
Dedrick, R.L., Zaharko, D.S. and Lutz, R.J.: Transport and binding of methotrexate in vivo. J. Pharm. Sci. 62, 1973b, 882.
Dedrick, R.L., Myers, C.E., Bungay, P.M. and DeVita, V.T.: Pharmacokinetic rationale for peritoneal drug administration in the treatment of ovarian cancer. Cancer Treatm. Rep. 62, 1978, 1.
DiFronzo, G., Gambettam R.A. and Lenaz, L.: Distributions and metabolism of adriamycin in mice. Comparison with daunomycin. Rev. Eur. Etud. Clin. Biol. 16, 1971, 572.
DiFronzo, G., Lenaz, L. and Bonadonna, G.: Distribution and excretion of adriamycin in man. Biomedicine Express 19, 1973, 169.
DiMarco, A., Gaetani, M. and Scarpinato, B.: Adriamycin (NSC-123127). A new antibiotic with antitumor activity. Cancer Chemother. Rep. 53, 1969, 33.
DiMarco, A., Zunino, F., Silvestrini, R. et al.: Interaction of some daunomycin derivates with deoxyribonucleic acid and their biological activity. Biochem. Pharmacol. 20, 1971, 1323.
DiMarco, A.: Adriamycin: the therapeutic activity on experimental tumors. In: Int. Symp. on Adriamycin (Eds. S.K. Carter, A. DiMarco and M. Ghione). Springer Verlag, New York, 1972, p. 53.
DiMarco, A., Casazza, A.M., Dasdia, T. et al.: Cytotoxic antiviral and antitumor activity of some derivates of daunomycin (NSC-82151). Cancer Chemother. Rep. 57, 1973, 269.
DiMarco, A.: Interactions of oncostatic agents with molecular mechanisms involved in transformation and proliferation. Eur. Assoc. Cancer Res., 2nd Meeting, Heidelberg, 1973.

DiMarco, A.: Adriamycin (NSC-123127): Mode and mechanism of action. Cancer Chemother. Rep. 6. 1975, 91.
DiMarco, A.: Mechanisms of action and mechanisms of resistance to antineoplastic agents that bind to DNA. In: Fundamentals in Cancer Chemotherapy (Ed. F.M. Schabel Jr.), Karger, Basel, 1978, 216.
Donelli, M.G., Martini, A., Colombo, T., Bossi, A. and Garattini, S.: Heart levels of adriamycin in normal and tumor-bearing mice. Europ. J. Cancer 12, 1976, 913.
Donelli, M.G., Colombo, T., Broggini, M. and Garattini, S.: Differential distribution of antitumor agents in primary and secondary tumors. Cancer Treatm. Rep. 61, 1977, 1319.
Dusonchet, L., Gebbia, N. and Gerbasi, F.: Spectrophotofluorometric characterization of adriamycin, a new antitumor drug. Pharmacol. Res. Communic. 3, 1971, 55.
Eksborg, S.: Reversed-phase liquid chromatography of adriamycin and daunorubicin and their hydroxyl metabolites adriamycinol and daunorubicinol. J. Chromatogr. 149, 1978, 225.
Eijkhoff, P.: System identification: state and parameter estimation. Whiley, New York, 1974.
Fested, R.L., Gee, M. and Bachur, N.R.: Rat liver daunorubicin reductase, an aldo-keto reductase. J. Biol. Chem. 249, 1974, 3672.
Finkel, J.M., Knapp, K.T. and Mulligan, L.T.: Fluorometric determination of serum levels and urinary excretion of daunomycin in mice and rats. Cancer Chemother. Rep. 53, 1969, 159.
Frei III, E.: Advances in the treatment of acute leukemia. Plenary Lecture XVIII Congress of Intern. Hematology, Paris, 1978.
Freireich, E.J., Gehan, E.A., Rall, D.P., Schmidt, L.H., Skipper,H.E.: Quantitative comparison of toxicity of anticancer agents in mouse, rat, dog, monkey and man. Cancer Chemother. Rep. 50, 1966, 219.
Gale, R.P. and Cline, M.J.: High remission-induction rate in acute myeloid leukaemia. Lancet 1, 1977, 497.
Gale, R.P.: Advances in the treatment of acute myelogenous leukemia. New Engl. J. Med. 300, 1979, 1189.
Glucksberg, H., Buckner, D.C., Fefer, A. et al.: Combination chemotherapy for acute nonlymphoblastic leukemia in adults. Cancer Chemother. Rep. 59, 1975, 1131.
Hagenbeek, A.: Extracorporeal irradiation of the blood in a rat leukemia model. Thesis, Rotterdam University, 1977.
Harris, P.A. and Gross, J.F.: Preliminary pharmacokinetic model for adriamycin (NSC-123127). Cancer Chemother. Rep. 59, 1975, 819.
Hartmann, G., Goller, H., Koschel, K. et al.: Hemmung der DNA-abhangigen RNA- and DNA-synthese durch Antibiotica. Biochem. Z.1964, 341.
Hoeschele, J.D. and Van Camp, L.: Whole body counting and the distribution of cis - 195 m - (Pt(NH3)2Cl2) in the major organs of Swiss white mice. In: Advances in antimicrobial and antineoplastic chemotherapy. 2, University Park Press, Baltimore, Md., 1972, 241.
Holland, J.F., Glidewell, O.J., Ellison, R.R. et al.: Acute myelocytic leukemia. Arch. Intern. Med. 136, 1976, 1377.
Kim, S.H. and Kim, J.H.: Lethal effect of adriamycin on the division cycle of HeLa cells. Cancer Res. 32, 1972, 323.
Kohn, K.W.: Abstract, Anal. Chem. 33, 1961, 862.
Koschel, K., Hartmann, G., Kersten, W. et al.: Die Wirkung des Chromomycins und Einiger Anthracyclinantibiotica auf die DNA-Abhangige nucleinsaure Synthese. Bioch. Z. 344, 1966, 76.
Krishan, A.: Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J. Cell. Biol. 66, 1975, 188.
Lenaz, L. and Page, J.A.: Cardiotoxicity of adriamycin and related anthracyclines. Cancer Treatm. Rev. 3, 1976, 111.
Loeb, L.A. and Agarwal, S.S.: DNA polymerase. Correlation with DNA replication during transformation of human lymphocytes. Exptl. Cell Res. 66, 1971, 299.

Lutz, R.J., Dedrick, R.L., Straw, J.A. et al.: The kinetics of methotrexate distribution in spontaneous canine lymphosarcoma. J. Pharmacokin. Biopharm. 3, 1975, 77.
Mantovani, A., Vecchi, A., Tagliabre, A. and Spreafico, F.: The effects of adriamycin and daunomycin on antitumoral immune effector mechanisms in an allogeneic system. Eur. J. Cancer 12, 1976a, 371.
Mantovani, A., Tagliabue, A., Vecchi, A. and Spreafico, F.: Effects of adriamycin and daunomycin on spleen cell populations in normal and tumor allografted mice. Eur. J. Cancer 12, 1976b, 381.
Mason, J.W., Bristow, M.R., Billingham, M.E. and Daniels, J.R.: Invasive and noninvasive methods of assessing adriamycin cardiotoxic effects in man: Superiority of histopathologic assessement using endomyocardial biopsy. Cancer Treatm. Rep. 62, 1978, 857.
McCredie, K.B., Bodey, G.A., Gutterman, J.U. et al.: Sequential adriamycin-Ara-C (AD-OAP) for remission induction (RI) of adult acute leukemia (AAL). Proc. Am. Assoc. Cancer Res. 15, 1974, 62.
McCredie, K.B., Hewlett, J.S., Gehan, E.A., Freireich, E.J.: Chemoimmunotherapy of adult acute leukemia (CIAL). Cancer Res. 18, 1977, 127.
Meriwether, W.D. and Bachur, N.R.: Inhibition of DNA and RNA metabolism by daunorubicin and adriamycin in L1210 mouse leukemia. Cancer Res. 32, 1972, 1137.
Myers, C.E., McGuire, W.P., Liss, R.H. et al.: Adriamycin: the role of lipid peroxidation in cardiac toxicity and tumor response. Science 197, 1977, 165.
Michaelis, L. and Menten, M.L.: Die Kinetik der Invertinwirkung. Biochem. Z. 49, 1913, 333,
Momparler, R.L., Karon, M., Siegel, S.E. and Avila, F.: Effect of Adriamycin on DNA, RNA and protein synthesis in cell-free systems and intact cells. Cancer Res. 36, 1976, 2891.
Morrison, P.F., Lincoln, T.L. and Aroesty, J.: The disposition of ara-C and its metabolites: A pharmacokinetic simulation. Cancer Chemother. Rep. 59, 1975, 861.
Mulder, J.A., Jonkers, H.L., Horsten, J.J. et al.: Analysis of aircraft performance, stability and control measurements. AGARD Lecture Series, 0.104, on Parameter Identification, Delft, London, 1979.
Munck-Petersen, B., Tyrsted, G. and Dupont, B.: The deoxyribonucleoside 5'-triphosphate (α ATP and α TTP) pool in phytohemagglutinin-stimulated and non-stimulated human lymphocytes. Exptl. Cell. Res. 79, 1973, 249.
Pegg, D.E.: Bone marrow transplantation. Lloyd-Luke (Medical Books) Ltd., 1966, 4.
Preisler, H.D., Bjornsson, S. and Henderson, E.S.: Adriamycin-cytosin arabinoside therapy for adult acute myelocytic leukemia. Cancer Treat. Rep. 61, 1977, 89.
Rai, K.R., Holland, J.F. and Glidewell, O.J.: Improvement in remission induction therapy of acute myelocytic leukemia. Proc. Am. Assoc. Cancer Res. Am. Soc. Clin. Oncol. 16, 1975, 265.
Reich, S.D., Steinberg, F., Bachur, N.R., Riggs Jr., C.E., Goebel, R. and Berman, M.: Mathematical model for adriamycin (doxorubicin) pharmacokinetics. Cancer Chemother. Pharmacol. 3, 1979, 125.
Riehm, H. and Biedler, J.L.: Cellular resistance to daunomycin in Chinese hamster cells in vitro. Cancer Res. 31, 1971, 409.
Roper, P.R. and Drewinko, B.: Comparison of in vitro methods to determine drug-induced cell lethality. Cancer Res. 36, 1976, 2182.
Sandberg, J.S., Howsden, F.L., DiMarco, A. et al.: Comparison of the antileukemic effect in mice of adriamycin (NSC 123127) with daunomycin (NSC 82151). Cancer Chemother. Rep. 54, 1970, 1.
Schwartz, H.S.: A fluorometric assay for daunomycin and adriamycin in animal tissues. Biochem. Medicine 7, 1973, 396.

Schwartz, H.S. and Kanter, P.M.: Cell interactions: Determinants of selective toxicity of adriamycin (NSC 123127) and daunorubicin (NSC 82151). Cancer Chemother. Rep. 6, 1975, 107.
Sieber, S.M.: The action of antitumor agents. A double-edged sword? Med. Pediat. Oncology 3, 1977, 123.
Silvestrini, R., DiMarco, A. and Dasdia, T.: Interference of daunomycin with metabolic events of the cell cycle in synchronized cultures of rat fibroblasts. Cancer Res. 30, 1970, 966.
Solcia, E., Ballerini, L., Bellini, O., Sala, L. and Bertazzoli, C.: Mammary tumors induced in rats by adriamycin and daunomycin. Cancer Res. 38, 1978, 1444.
Somberg, J., Cagin, N., Levitt, B., Bonnous, H., Ready, P., Leonard, D. and Anagnostopoulos, C.: Blockade of tissue uptake of the antineoplastic agent doxorubicin. J. Pharmacol. Exp. Therap. 204, 1978, 226.
Sonneveld, P.: Effect of α-tocopherol on the cardiotoxicity of adriamycin in the rat. Cancer Treat. Rep. 62, 1978, 1033.
Teorell, T.: Kinetics of distribution of substances administered to the body. II. The intravascular modes of administration. Arch. Int. Pharmacodyn. 57, 1937, 226.
Takanashi, S. and Bachur, N.R.: New adriamycin and daunorubicin metabolites in human urine. Proc. Am. Assoc. Cancer Res. and Asco. 15, 1974, 76.
Talbot, S.A. and Gessner, U.: Systems physiology. Wiley, New York, 1973, p. 9.
Tan, C., Etabanus, E., Wollner, N. et al.: Adriamycin, an antitumor antibiotic in the treatment of neoplastic disease. Cancer 32, 1973, 9.
Till, J.E. and McCulloch, E.A.: A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. 14, 1961, 213.
Tobey, R.A.: Effects of cytosine arabinoside, daunomycin, mithramycin, azacytidine, adriamycin and camptothecin on mammalian cell cycle traverse. Cancer Res. 32, 1972, 2720.
Tobey, R.A., Crissman, H.A. and Oka, M.S.: Arrested and cycling CHO cells as a kinetic model: Studies with adriamycin. Cancer Treat. Rep. 60, 1976, 1829.
Tyrsted, G., Munck-Petersen, B. and Cloos, L.: DNA polymerase activity in phytohaemagglutinin-stimulated and non-stimulated human lymphocytes. Exptl. Cell Res. 77, 1973, 415.
Vecchi, A., Cairo, M., Mantovani, A., Sironi, M. and Spreafico, F.: Comparative antineoplastic activity of adriamycin and N-trifluoroacetuladriamycin-14-valerate. Cancer Treat. Rep. 62, 1978, 111.
Wagner, J.G.: Fundamentals of clinical pharmacokinetics. Drug Intelligence Publications Inc. Hamilton, Ill.62341, 1975.
Wang, J.J., Chervinsky, D.S. and Rosen, J.M.: Comparative biochemical studies of adriamycin and daunomycin in leukemic cells. Cancer Res. 32, 1972, 511.
Wantzin, G.H. and Killmann, S.A.: Nuclear labelling of leukaemic blast cells with tritiated thymidine triphosphate after daunomycin. Europ. J. Cancer 13, 1977, 647.
Watson, E. and Chan, K.: Rapid analytic method for adriamycin and metabolites in human plasma by a thin-film fluorescence scanner. Cancer Treatm. Rep. 60, 1976, 1611.
Weil, M., Glidewell, O.J., Jacquillat, C. et al.: Daunorubicin in the therapy of acute granulocytic leukemia. Cancer Res. 33, 1973, 921.
Widmark, E. and Tanberg. J.: Ueber die Bedingungen für die Akkumulation indifferenter Narkotika, Theoretische Berechnungne. Biochem. Z. 147, 1924, 358.
Wilbur, J., Etaubanas, E., Long, T. et al.: 4-Drug therapy and irradiation in primary and metastatic osteogenic sarcoma. In: Proc. Am. Assoc. Cancer Res. 15, 1974, 188.

Wilkinson, P.M., Israel, M., Pegg, W.J. and Frei III, E.: Comparative metabolism and excretion of adriamycin in man, monkey and rat. Cancer Chemother. Pharmacol. 2, 1979, 121.

Wilson, H.E., Bodey, G.P., Moon, T.E., Amare, M., Bottomley, R., Haut, A., Hewlett, J.S., Morrison, F. and Saiki, J.H.: Adriamycin therapy in previously treated adult acute leukemia. Cancer Treatm. Rep. 61, 1977, 905.

Yates, J.W., Wallace, Jr., H.J., Ellison, R.R. et al.: Cytosine arabinoside (NSC-63878) and daunorubicin (NSC-83142) therapy in acute nonlymphocytic leukemia. Cancer Chemother. Rep. 57, 1973, 485.

Yesair, D.W., Schwartzbach, E., Shuck, D., Denine, E.P. and Asbell, M.A.: Comparative pharmacokinetics of daunomycin and adriamycin in several animal species. Cancer Res. 32, 1972, 1177.

Zbinden, G. and Brandle, E.: Toxicologic screening of Daunorubicin (NSC-82151), Adriamycin (NSC.123127) and their derivatives in rats. Cancer Chemother. Rep. 59 (parts) 707, 1975.

Zunino, F., Gumbetta, R., DiMarco, A. et al.: Interaction of daunomycin and its derivates with DNA. Biochim. Biophys. Acta 277, 1972, 489.

Zunino, F., DiMarco, A., Zaccara, A. et al.: The inhibition of RNA polymerase by daunomycin. Chem. Biol. Interact. 9, 1974, 25.

Zunino, F., Gambetta, R., DiMarco, A. et al.: A comparison of the effects of daunomycin and adriamycin on various DNA polymerases. Cancer Res. 35, 1975, 754.

ABBREVIATIONS

AML	acute myelocytic leukaemia
ara-C	arabinoside cytosine
BN	Brown Norway rat
BNML	acute myelocytic leukaemia in the Brown Norway rat
CFU-S	colony forming unit spleen
DNA	deoxyribonucleic acid
^{59}Fe	59-Iron
HSC	pluripotent haemopoietic stem cell
^{3}H-TdR	tritiated thymidine
^{3}H-UdR	tritiated uridine
i.v.	intravenous
LCFU-S	leukaemic colony forming unit spleen
L1210	L1210 mouse leukaemia
LI	^{3}H-TdR labelling index
MI	^{3}H-TdR mitotic index
PHA	phytohaemagglutinine
PI	propidium iodide
RNA	ribonucleic acid
SD	standard deviation
SE	standard error
T 1/2	halving time
Vd	volume distribution

ACKNOWLEDGEMENTS

The author expresses his gratitude to the scientific and technical staff of the REPGO-TNO institutes for their stimulating support and for providing the facilities to perform the present study. In particular he is indebted to:

- Ir. J.A. Mulder (Dept. of Aerospace Engineering, Delft University of Technology) for his friendship and continuous interest and cooperation with the study.
- Mr. J.H.N. Vollebregt for his expert technical assistance and good fellowship.
- Dr. A. Hagenbeek, Dr. M.Aglietta, Dr. L.P.Colly and Ir. F.W.Schultz, who were participants in the experimental leukaemia project, for the stimulating discussions whether or not about science.
- Dr. G.J. van den Engh, Drs. J.H. Mulder, Prof.Dr. L.M. van Putten, Dr. J.W.M. Visser and Mr. N. Vreeken for their expert advices and assistance.
- Miss A. Tönss, Mr. A. Martens, Miss B. van Ham, Miss C.van Marrewijk, Mrs. S. Knaan, Mr. R. Korving, Mrs. W. Asscherman and Miss A. Elsgeest who created the typical atmosphere of the bone marrow room.
- Mrs. M.van der Sman and Miss D. van der Velden for accurately typing the manuscript and Mr. J.Ph. de Kler for preparing the figures.
- Dr. A.C. Ford for editing the English text and Mr. P.G.M. van Rossum for collecting the literature.

The work described in this thesis was supported by the "Koningin Wilhelmina Fonds" of the Dutch National Cancer League.

CURRICULUM VITAE

Pieter Sonneveld was born in Schiedam in 1951. After completing his secondary education (Gymnasium-β) in 1970, he studied medicine at the University of Utrecht (1970-1971) and the Erasmus University in Rotterdam (1971-1976). In 1973, he participated in a historical study on leukemia research with Prof.Dr. M.J. de Vries at the Department of Pathology. After he was registered M.D. in 1976, he worked at the Rutgersstichting on birth control. In 1977, he was certified by the American Examination Council for Foreign Medical Graduates. In 1977, he joined the Staff of the Radiobiological Institute TNO in Rijswijk (director: Prof.Dr. D.W. van Bekkum). This thesis describes work performed at this Institute.